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(71) Applicant: **KYOWA HAKKO KOGYO CO., LTD.**
Chiyoda-ku, Tokyo 100-8185 (JP)

(72) Inventors:
• **MIYAJI, Hiromasa** Pharmaceu. Res. Institute
Nagaizumi Sunto Shizuoka 411-8731 (JP)
• **HARUOKA, Motoko**
Pharmaceutical Research Institute
Nagaizumi Sunto Shizuoka 411-8731 (JP)

- **NAGATA, Hiroyuki**
Tokyo Research Laboratories
Machida-shi Tokyo 194-8533 (JP)
- **OTA, Toshio** **Tokyo Research Laboratories**
Machida-shi Tokyo 194-8533 (JP)
- **KAWABATA, Ayako**
Tokyo Research Laboratories
Machida-shi Tokyo 194-8533 (JP)
- **SUGANO, Sumio**
Suginami-ku, Tokyo 167-0052 (JP)
- **NAKAMURA, Yusuke**
Yokohama-shi, Kanagawa 225-0011 (JP)

(74) Representative: **Denholm, Anna Marie et al**
D Young & Co.,
21 New Fetter Lane
London EC4A 1DA (GB)

(54) **POLYPEPTIDE HAVING PHOSPHOLIPASE A₂ ACTIVITY**

(57) The present invention relates to a novel phospholipase A₂ polypeptide, DNA encoding the polypeptide, a vector comprising the DNA, a transformant transformed with the vector, and a process for producing the phospholipase A₂ polypeptide. The present invention also relates to a method of utilizing the polypeptide, e.g., a method of screening for a compound having agonist or antagonist activity by using the polypeptide or an antibody to the polypeptide, and a pharmaceutical comprising the polypeptide or an antibody to the polypep-

ptide. The present invention further relates to a polypeptide inhibiting the phospholipase A₂ activity of a phospholipase A₂ polypeptide (hereinafter referred to as inhibitor polypeptide), DNA encoding the inhibitor polypeptide, a vector comprising the DNA encoding the inhibitor polypeptide, a transformant transformed with the vector, a pharmaceutical comprising the inhibitor polypeptide, and a process for producing the inhibitor polypeptide.

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DescriptionTechnical Field

[0001] The present invention relates to a novel phospholipase A₂ polypeptide, DNA encoding the polypeptide, a vector comprising the DNA, a transformant transformed with the vector, and a process for producing the phospholipase A₂ polypeptide. The present invention also relates to a method of utilizing the polypeptide, e.g., a method of screening for a compound having agonist or antagonist activity by using the polypeptide or an antibody to the polypeptide, and a pharmaceutical comprising the polypeptide or an antibody to the polypeptide. The present invention further relates to a polypeptide inhibiting the phospholipase A₂ activity of a phospholipase A₂ polypeptide (hereinafter sometimes referred to as inhibitor polypeptide), DNA encoding the inhibitor polypeptide, a vector comprising the DNA encoding the inhibitor polypeptide, a transformant transformed with the vector, a pharmaceutical comprising the inhibitor polypeptide, and a process for producing the inhibitor polypeptide.

Background Art

[0002] "Phospholipase" is a general term for enzymes hydrolyzing the ester bonds in glycerophospholipid, which is a biomembrane component. Phospholipase is classified into phospholipase A₁, phospholipase A₂, phospholipase B, phospholipase C and phospholipase D, according to the position of hydrolysis.

[0003] Phospholipase A₂ hydrolyzes the ester bond at the sn-2-position in glycerophospholipid to form fatty acid and lysophospholipid. Among the released fatty acids, arachidonic acid is metabolized into prostaglandin and leukotriene via cyclooxygenase and 5-lipoxygenase, respectively. Lysophospholipid is also metabolized into a platelet-activating factor.

[0004] That is, phospholipase A₂ is considered as an enzyme initiating the formation of such lipid mediators. Inhibitors of cyclooxygenase and 5-lipoxygenase have already been used clinically as antiinflammatory drugs, and therefore, an inhibitor of phospholipase A₂ located upstream of them is expected to be a potent antiinflammatory drug capable of simultaneously blocking the formation of them.

[0005] Phospholipase A₂ is broadly classified into three subfamilies, i.e., secretory phospholipase A₂, cytoplasmic phospholipase A₂ and Ca²⁺ independent phospholipase A₂, according to the structure and properties [J. Biol. Chem., 269, 13057 (1994)].

[0006] As to cytoplasmic phospholipase A₂, three subtypes, α, β and γ, are known. Cytoplasmic phospholipase A₂α, A₂β and A₂γ are enzymes respectively having the molecular weight of 85 kilodaltons, 110 kilodaltons and 60 kilodaltons, all of which are generally expressed in most tissues. Arginine at position 200, serine at position 228 and aspartic acid at position 549 of the amino acid sequence of cytoplasmic phospholipase A₂α are essential for its activity [J. Biol. Chem., 271, 19225 (1996)] and are conserved in cytoplasmic phospholipase A₂β and A₂γ.

[0007] Cytoplasmic phospholipase A₂α and A₂β have C2 domain in the N-terminal region and Ca²⁺-dependently bind to phospholipid membrane via the domain. Cytoplasmic phospholipase A₂γ does not have C2 domain [J. Biol. Chem., 273, 21926 (1998); J. Biol. Chem., 274, 8823 (1999); J. Biol. Chem., 274, 17063 (1999)].

[0008] Cytoplasmic phospholipase A₂α is considered to participate in formation of lipid mediators by stimulus [J. Biol. Chem., 272, 16709 (1997)]. Physiological functions of cytoplasmic phospholipase A₂β and A₂γ have not been clarified yet.

[0009] It can be assumed that production of lipid mediators is concerned in the occurrence and progress of some diseases such as inflammation and allergy. In order to prevent or treat such diseases, there exists a need for inhibitors specific to phospholipase A₂ subtype which is concerned in the diseases.

[0010] On the contrary, in view of the report that phospholipase A₂ acts as a promoter of insulin secretion in pancreas [Biochimica et Biophysica Acta, 1390, 301 (1998); Biochemical Society Transactions, 25, 213S (1997); Biochemical Pharmacology, 53, 1077 (1997)], it is expected that enhancement of phospholipase A₂ activity is effective for the prevention or treatment of diabetes.

[0011] In either case of inhibiting or enhancing phospholipase A₂ activity, use of nonspecific chemicals is undesirable because of effect on the phospholipid metabolism in tissues and cells other than target tissues and cells.

[0012] However, the expression of cytoplasmic phospholipase A₂α, β and γ is ubiquitous, and no tissue- or cell-specific cytoplasmic phospholipase A₂ has so far been known.

[0013] Therefore, in order to attain the object of the present invention, it is necessary to identify and isolate phospholipase A₂ concerned in specific diseases.

[0014] In the case of cytoplasmic phospholipase A₂, purification and isolation from tissues or cells is not easy because it exists only in extremely small amounts. The limitation of currently employed purification methods and the difficulty in confirming that a single purified enzyme preparation has been obtained hinder the isolation of a novel subtype using conventional enzymological techniques.

[0015] Accordingly, it is expected that if a novel tissue- or cell-specific phospholipase subtype can be found and prepared in large amounts using recombinant DNA techniques, the use of such phospholipase subtype will enable the development of more specific and safer inhibitors.

5 Disclosure of the Invention

[0016] An object of the present invention is to provide a novel phospholipase A₂ polypeptide and DNA encoding the phospholipase A₂ polypeptide.

10 [0017] Another object of the present invention is to provide a pharmaceutical for the diagnosis, prevention or treatment of asthma, ischemic diseases, arthritis, rheumatism, sepsis, dermatitis, arteriosclerosis, pain, Parkinson disease, Alzheimer disease, malignant tumor, nephritis, diabetes or ischemic reperfusion injury by using the phospholipase A₂ polypeptide, a polypeptide inhibiting the activity of the phospholipase A₂ polypeptide, an antibody recognizing the phospholipase A₂ polypeptide, or the like.

15 [0018] The present inventors prepared a cDNA library from human small intestine and carried out analysis of nucleotide sequences at random. The obtained nucleotide sequences were analyzed by using BLAST SEARCH homology search software, and as a result, a sequence was found which was recognized as homologous to C2 domain of human cytoplasmic phospholipase A₂β (GenBank; AAC78836). The inventors determined the entire nucleotide sequence of the clone, and on the basis of the nucleotide sequence, cloned cDNA completely containing the region homologous to cytoplasmic phospholipase A₂ including catalytic domain from a human kidney cDNA library. By determining and
20 analyzing the entire nucleotide sequence of the clone, the present invention has been completed.

[0019] The present invention relates to the following (1) to (57).

(1) A polypeptide having an amino acid sequence selected from the group consisting of the amino acid sequences shown in SEQ ID NOS: 1, 22, 26 and 38.

25 (2) A polypeptide consisting of an amino acid sequence wherein one or more amino acid residues are deleted, substituted or added in an amino acid sequence selected from the group consisting of the amino acid sequences shown in SEQ ID NOS: 1, 22, 26 and 38 and having phospholipase A₂ activity.

30 (3) A polypeptide consisting of an amino acid sequence which has 60% or more homology to an amino acid sequence selected from the group consisting of the amino acid sequences shown in SEQ ID NOS: 1, 22, 26 and 38 and having phospholipase A₂ activity.

(4) A DNA encoding the polypeptide according to any of the above (1) to (3).

35 (5) A DNA having a nucleotide sequence selected from the group consisting of the nucleotide sequences shown in SEQ ID NOS: 2, 23, 27 and 39.

40 (6) A DNA which hybridizes to a DNA consisting of a nucleotide sequence complementary to a nucleotide sequence selected from the group consisting of the nucleotide sequences shown in SEQ ID NOS: 2, 23, 27 and 39 under stringent conditions and which encodes a polypeptide having phospholipase A₂ activity.

(7) A recombinant vector comprising the DNA according to any of the above (4) to (6).

45 (8) A transformant carrying the recombinant vector according to the above (7).

(9) The transformant according to the above (8), wherein the transformant is selected from the group consisting of a microorganism, an animal cell, a plant cell and an insect cell.

50 (10) The transformant according to the above (9), wherein the microorganism is a microorganism belonging to the genus Escherichia.

(11) The transformant according to the above (9), wherein the microorganism is Escherichia coli JM109/p5269+C5 (FERM BP-7281).

55 (12) A process for producing a polypeptide having phospholipase A₂ activity, which comprises culturing the transformant according to any of the above (8) to (11) in a medium, allowing the polypeptide having phospholipase A₂ activity to form and accumulate in the culture, and recovering the polypeptide from the culture.

(13) An oligonucleotide selected from the group consisting of a sense oligonucleotide having a nucleotide sequence identical with a sequence of 5 to 60 consecutive nucleotides in the nucleotide sequence of the DNA according to any of the above (4) to (6), an antisense oligonucleotide having a nucleotide sequence complementary to that of said sense oligonucleotide, and a derivative of said sense oligonucleotide or antisense oligonucleotide.

(14) An oligonucleotide consisting of a nucleotide sequence selected from the group consisting of the nucleotide sequences shown in SEQ ID NOS: 13, 14, 28, 29, 30, 31, 46 and 47.

(15) The oligonucleotide according to the above (13), wherein the oligonucleotide derivative is selected from the group consisting of an oligonucleotide derivative wherein the phosphodiester bond in an oligonucleotide is converted to a phosphorothioate bond, an oligonucleotide derivative wherein the phosphodiester bond in an oligonucleotide is converted to an N3'-P5' phosphoamidate bond, an oligonucleotide derivative wherein the ribose-phosphodiester bond in an oligonucleotide is converted to a peptide-nucleic acid bond, an oligonucleotide derivative wherein the uracil in an oligonucleotide is substituted by C-5 propynyluracil, an oligonucleotide derivative wherein the cytosine in an oligonucleotide is substituted by C-5 propynylcytosine, an oligonucleotide derivative wherein the cytosine in an oligonucleotide is substituted by phenoxazine-modified cytosine, an oligonucleotide derivative wherein the ribose in DNA is substituted by 2'-O-propylribose, and an oligonucleotide derivative wherein the ribose in an oligonucleotide is substituted by 2'-methoxyethoxyribose.

(16) A method for detecting an mRNA encoding the polypeptide according to any of the above (1) to (3), which comprises using the oligonucleotide according to any of the above (13) to (15).

(17) A method for inhibiting the expression of the polypeptide according to any of the above (1) to (3), which comprises using the oligonucleotide according to any of the above (13) to (15).

(18) An antibody recognizing the polypeptide according to any of the above (1) to (3).

(19) A method for immunological detection of the polypeptide according to any of the above (1) to (3), which comprises using the antibody according to the above (18).

(20) A method for immunohistochemical staining of the polypeptide according to any of the above (1) to (3), which comprises using the antibody according to the above (18).

(21) An immunohistochemical staining agent comprising the antibody according to the above (18).

(22) A method for screening for a compound varying the phospholipase A₂ activity of the polypeptide according to any of the above (1) to (3), which comprises contacting said polypeptide with a test sample, and measuring the phospholipase A₂ activity of said polypeptide.

(23) A method for screening for a compound varying the expression level of the polypeptide according to any of the above (1) to (3), which comprises contacting cells expressing said polypeptide with a test sample, and detecting the expression level of said polypeptide.

(24) The method according to the above (23), wherein said detection of the expression level of said polypeptide is detection of an mRNA encoding the polypeptide according to any of the above (1) to (3) using the method according to the above (16).

(25) The method according to the above (23), wherein said detection of the expression level of said polypeptide is detection of the polypeptide using the method according to the above (19).

(26) The method according to the above (22), wherein said variation of the phospholipase A₂ activity of the polypeptide according to any of the above (1) to (3) is an increase in the phospholipase A₂ activity of said polypeptide.

(27) The method according to the above (22), wherein said variation of the phospholipase A₂ activity of the polypeptide according to any of the above (1) to (3) is a decrease in the phospholipase A₂ activity of said polypeptide.

(28) The method according to any of the above (23) to (25), wherein said variation of the expression of the polypep-

tide according to any of the above (1) to (3) is an increase in the expression level of said polypeptide.

(29) The method according to any of the above (23) to (25), wherein said variation of the expression of the polypeptide according to any of the above (1) to (3) is a decrease in the expression level of said polypeptide.

(30) A compound which is obtainable by the method according to any of the above (22) to (29).

(31) A promoter DNA regulating the transcription of a DNA encoding the polypeptide according to any of the above (1) to (3).

(32) A method for screening for a compound varying the efficiency of transcription of a DNA encoding the polypeptide according to any of the above (1) to (3), which comprises contacting a transformant carrying a plasmid containing the promoter DNA according to the above (31) and a reporter gene ligated downstream of said promoter DNA with a test sample, and measuring the content of the translation product of said reporter gene.

(33) The method according to the above (32), wherein the reporter gene is a gene selected from the group consisting of a chloramphenicol acetyltransferase gene, a β -galactosidase gene, a luciferase gene, a β -glucuronidase gene, an aequorin gene and a green fluorescent protein gene.

(34) The method according to the above (32) or (33), wherein said variation of the efficiency of transcription of a DNA encoding the polypeptide according to any of the above (1) to (3) is an increase in the efficiency of transcription of said DNA.

(35) The method according to the above (32) or (33), wherein said variation of the efficiency of transcription of a DNA encoding the polypeptide according to any of the above (1) to (3) is a decrease in the efficiency of transcription of said DNA.

(36) A compound which is obtainable by the method according to the above (32) to (35).

(37) A polypeptide consisting of an amino acid sequence wherein a part or the whole of the amino acid sequence of the active domain is deleted in the amino acid sequence of the polypeptide according to any of the above (1) to (3).

(38) A polypeptide consisting of the amino acid sequence shown in SEQ ID NO: 3.

(39) A polypeptide consisting of an amino acid sequence wherein one or more amino acid residues are deleted, substituted or added in the amino acid sequence shown in SEQ ID NO: 3 and having the activity of inhibiting phospholipase A_2 activity.

(40) A polypeptide consisting of an amino acid sequence which has 60% or more homology to the amino acid sequence shown in SEQ ID NO: 3 and having the activity of inhibiting phospholipase A_2 activity.

(41) A DNA encoding the polypeptide according to any of the above (37) to (40).

(42) A DNA having the nucleotide sequence shown in SEQ ID NO: 4.

(43) A DNA which hybridizes to a DNA consisting of a nucleotide sequence complementary to the nucleotide sequence shown in SEQ ID NO: 4 under stringent conditions and which encodes a polypeptide having the activity of inhibiting phospholipase A_2 activity.

(44) A recombinant vector comprising the DNA according to any of the above (41) to (43).

(45) A transformant carrying the recombinant vector according to the above (44).

(46) The transformant according to the above (45), wherein the transformant is selected from the group consisting of a microorganism, an animal cell, a plant cell and an insect cell.

(47) A process for producing a polypeptide having the activity of inhibiting phospholipase A_2 activity, which comprises culturing the transformant according to the above (45) or (46) in a medium, allowing the polypeptide having

the activity of inhibiting phospholipase A₂ activity to form and accumulate in the culture, and recovering the polypeptide from the culture.

(48) A pharmaceutical for the diagnosis, prevention or treatment of a disease in which the polypeptide according to any of the above (1) to (3) is concerned, which comprises, as an active ingredient, a compound varying the phospholipase A₂ activity of said polypeptide.

(49) A pharmaceutical for the diagnosis, prevention or treatment of a disease in which the polypeptide according to any of the above (1) to (3) is concerned, which comprises said polypeptide as an active ingredient.

(50) A pharmaceutical for the diagnosis, prevention or treatment of a disease in which the polypeptide according to any of the above (1) to (3) is concerned, which comprises, as an active ingredient, the DNA according to any of the above (4) to (6).

(51) A pharmaceutical for the diagnosis, prevention or treatment of a disease in which the polypeptide according to any of the above (1) to (3) is concerned, which comprises, as an active ingredient, the polypeptide according to any of the above (37) to (40).

(52) A pharmaceutical for the diagnosis, prevention or treatment of a disease in which the polypeptide according to any of the above (1) to (3) is concerned, which comprises, as an active ingredient, the DNA according to any of the above (41) to (43).

(53) A pharmaceutical for the diagnosis, prevention or treatment of a disease in which the polypeptide according to any of the above (1) to (3) is concerned, which comprises, as an active ingredient, the oligonucleotide according to any of the above (13) to (15).

(54) A pharmaceutical for the diagnosis, prevention or treatment of a disease in which the polypeptide according to any of the above (1) to (3) is concerned, which comprises, as an active ingredient, the antibody according to the above (18).

(55) A pharmaceutical for the diagnosis, prevention or treatment of a disease in which the polypeptide according to any of the above (1) to (3) is concerned, which comprises, as an active ingredient, the compound according to the above (30) or (36).

(56) The pharmaceutical according to any of the above (48) to (55), wherein said disease in which said polypeptide is concerned is asthma, ischemic diseases, arthritis, rheumatism, sepsis, dermatitis, arteriosclerosis, pain, Parkinson disease, Alzheimer disease, malignant tumor, nephritis, diabetes or ischemic reperfusion injury.

(57) A pharmaceutical for the diagnosis, prevention or treatment of diabetes, which comprises, as an active ingredient, a compound obtainable by the method according to the above (28) or (34).

[0020] The polypeptides of the present invention include a polypeptide having an amino acid sequence selected from the group consisting of the amino acid sequences shown in SEQ ID NOS: 1, 22, 26 and 38.

[0021] The polypeptides of the present invention also include a polypeptide consisting of an amino acid sequence wherein one or more amino acid residues are deleted, substituted or added in an amino acid sequence selected from the group consisting of the amino acid sequences shown in SEQ ID NOS: 1, 22, 26 and 38 and having phospholipase A₂ activity, and a polypeptide comprising an amino acid sequence which has 60% or more homology to an amino acid sequence selected from the group consisting of the amino acid sequences shown in SEQ ID NOS: 1, 22, 26 and 38 and having phospholipase A₂ activity.

[0022] The polypeptide consisting of an amino acid sequence wherein one or more amino acid residues are deleted, substituted or added in an amino acid sequence selected from the group consisting of the amino acid sequences shown in SEQ ID NOS: 1, 22, 26 and 38 and having phospholipase A₂ activity and the polypeptide comprising an amino acid sequence which has 60% or more homology to an amino acid sequence selected from the group consisting of the amino acid sequences shown in SEQ ID NOS: 1, 22, 26 and 38 and having phospholipase A₂ activity can be obtained, for example, by introducing a site-directed mutation into DNA encoding the polypeptide having an amino acid sequence selected from the group consisting of the amino acid sequences shown in SEQ ID NOS: 1, 22, 26 and 38 by site-directed mutagenesis described in Molecular Cloning, A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press (1989) (hereinafter referred to as Molecular Cloning, Second Edition); Current Protocols in Molecular

Biology, Supplement 1-38, John Wiley & Sons (1987-1997) (hereinafter referred to as Current Protocols in Molecular Biology); Nucleic Acids Research, 10, 6487 (1982); Proc. Natl. Acad. Sci. USA, 79, 6409 (1982); Gene, 34, 315 (1985); Nucleic Acids Research, 13, 4431 (1985); Proc. Natl. Acad. Sci. USA, 82, 488 (1985); Proc. Natl. Acad. Sci. USA, 81, 5662 (1984); Science, 224, 1431 (1984); WO85/00817; Nature, 316, 601 (1985), etc.

[0023] The number of amino acid residues which are deleted, substituted or added is not particularly limited, but is within the range of 1 to dozens, preferably 1 to 20, more preferably 1 to 10, further preferably 1 to 5; such number of amino acid residues can be deleted, substituted or added by known methods such as the above site-directed mutagenesis.

[0024] In order that the polypeptide of the present invention may have phospholipase A₂ activity, it is necessary that the homology of its amino acid sequence to an amino acid sequence selected from the group consisting of the amino acid sequences shown in SEQ ID NOS: 1, 22, 26 and 38, as calculated by use of analysis software such as BLAST [J. Mol. Biol., 215, 403 (1990)] or FASTA [Methods in Enzymology, 183, 63 (1990)], is at least 60%, preferably 70% or more, more preferably 80% or more, further preferably 90% or more, particularly preferably 95% or more, most preferably 98% or more.

[0025] Further, in order that the polypeptide may have phospholipase A₂α activity, it is preferred that the amino acid residues corresponding to arginine at position 200, serine at position 228 and aspartic acid at position 549, which are considered to be essential for the activity of cytoplasmic phospholipase A₂α, are conserved.

[0026] The polypeptides of the present invention do not include known polypeptides.

[0027] The DNA encoding the polypeptide of the present invention (hereinafter referred to as DNA of the present invention) may have any nucleotide sequence so far as it encodes the polypeptide of the present invention described above. The DNAs of the present invention include DNA having a nucleotide sequence selected from the group consisting of the nucleotide sequences shown in SEQ ID NOS: 2, 23, 27 and 39.

[0028] The DNAs of the present invention also include DNA which hybridizes to DNA consisting of a nucleotide sequence complementary to a nucleotide sequence selected from the group consisting of the nucleotide sequences shown in SEQ ID NOS: 2, 23, 27 and 39 under stringent conditions and which has a nucleotide sequence encoding a polypeptide having phospholipase A₂ activity.

[0029] The above "DNA which hybridizes to DNA consisting of a nucleotide sequence complementary to a nucleotide sequence selected from the group consisting of the nucleotide sequences shown in SEQ ID NOS: 2, 23, 27 and 39 under stringent conditions and which has a nucleotide sequence encoding a polypeptide having phospholipase A₂ activity" refers to DNA which is obtained by colony hybridization, plaque hybridization, Southern hybridization, or the like using, as a probe, the DNA consisting of a nucleotide sequence complementary to a nucleotide sequence selected from the group consisting of the nucleotide sequences shown in SEQ ID NOS: 2, 23, 27 and 39. A specific example of such DNA is DNA which can be identified by carrying out hybridization at 65°C in the presence of 0.7 to 1.0 mol/l NaCl using a filter with colony- or plaque-derived DNA immobilized thereon and then washing the filter at 65°C with a 0.1 to 2-fold conc. SSC (saline-sodium citrate) solution (1-fold conc. SSC solution: 150 mmol/l sodium chloride and 15 mmol/l sodium citrate).

[0030] Hybridization can be carried out according to the methods described in laboratory manuals such as Molecular Cloning, Second Edition; Current Protocols in Molecular Biology; and DNA Cloning 1: Core Techniques, A Practical Approach, Second Edition, Oxford University Press (1995).

[0031] Specifically, the DNA capable of hybridization includes DNA having 80% or more homology, preferably 90% or more homology, more preferably 95% or more homology, particularly preferably 98% or more homology to a nucleotide sequence selected from the group consisting of the nucleotide sequences shown in SEQ ID NOS: 2, 23, 27 and 39 as calculated by use of analysis software such as BLAST or FASTA.

[0032] The DNAs of the present invention do not include known DNAs.

[0033] Some of the polypeptides in which a part or the whole of the active domain of the polypeptide of the present invention is deleted are polypeptides inhibiting the phospholipase A₂ activity of the polypeptide of the present invention. Such polypeptides inhibiting phospholipase A₂ activity (inhibitor polypeptides) are useful, as inhibitors specific to phospholipase A₂ subtype, for preventing or treating diseases of which the occurrence or progress is considered to involve the production of lipid mediators (e.g., inflammation and allergy).

[0034] The inhibitor polypeptides are polypeptides in which at least a part of the active domain containing amino acids essential for the activity of the polypeptide of the present invention is deleted. A specific example of the inhibitor polypeptides is a polypeptide having the amino acid sequence shown in SEQ ID NO: 3.

[0035] The inhibitor polypeptides include a polypeptide consisting of an amino acid sequence wherein one or more amino acid residues are deleted, substituted or added in the amino acid sequence shown in SEQ ID NO: 3 and having the activity of inhibiting phospholipase A₂ activity, and a polypeptide comprising an amino acid sequence which has 60% or more homology to the amino acid sequence shown in SEQ ID NO: 3 and having the activity of inhibiting phospholipase A₂ activity. Such polypeptides can be obtained, for example, by introducing a site-directed mutation into DNA encoding the polypeptide shown in SEQ ID NO: 3 using methods similar to the above-described methods for obtaining

the polypeptide of the present invention.

[0036] The number of amino acid residues which are deleted, substituted or added is not particularly limited, but is within the range of 1 to dozens, preferably 1 to 20, more preferably 1 to 10, further preferably 1 to 5; such number of amino acid residues can be deleted, substituted or added by known methods such as the above site-directed mutagenesis.

[0037] In order that the polypeptide of the present invention may have the activity of inhibiting phospholipase A₂ activity, it is necessary that the homology of its amino acid sequence to the amino acid sequence shown in SEQ ID NO: 3, as calculated by use of analysis software such as BLAST or FASTA, is at least 60%, preferably 70% or more, more preferably 80% or more, further preferably 90% or more, particularly preferably 95% or more, most preferably 98% or more.

[0038] The DNA encoding the inhibitor polypeptide may have any nucleotide sequence so far as it encodes the inhibitor polypeptide described above. A specific example of the DNA encoding the inhibitor polypeptide is DNA having the nucleotide sequence shown in SEQ ID NO: 4.

[0039] The DNAs of the present invention also include DNA which hybridizes to DNA consisting of a nucleotide sequence complementary to the nucleotide sequence shown in SEQ ID NO: 4 under stringent conditions and which has a nucleotide sequence encoding a polypeptide having the activity of inhibiting phospholipase A₂ activity.

[0040] The above "DNA which hybridizes to DNA consisting of a nucleotide sequence complementary to the nucleotide sequence shown in SEQ ID NO: 4 under stringent conditions and which has a nucleotide sequence encoding a polypeptide having the activity of inhibiting phospholipase A₂ activity" refers to DNA which can be identified by methods similar to the methods for identifying the DNA of the present invention using, as a probe, the DNA consisting of a nucleotide sequence complementary to the nucleotide sequence shown in SEQ ID NO: 4.

[0041] Specifically, the DNA capable of hybridization includes DNA having 80% or more homology, preferably 90% or more homology, more preferably 95% or more homology, particularly preferably 98% or more homology to the nucleotide sequence shown in SEQ ID NO: 4 as calculated by use of analysis software such as BLAST or FASTA.

[0042] The present invention is described in detail below.

[1] Acquisition of the DNA of the Present Invention and

Preparation of Oligonucleotides

[0043] Gene database and protein database searches are made for DNA encoding an amino acid sequence having homology to the amino acid sequence of human phospholipase A₂β (GenBank: AAC78836) by using a program utilizing Blast, the Smith-Waterman method, or the like, or Frame Search (Compugen) homology search software.

[0044] As the database, public databases such as GenBank and Swiss-Plot can be utilized.

[0045] Also useful are private databases which have been prepared by determining the nucleotide sequences of cDNA clones in a private cDNA library at random on a large scale and collecting the obtained sequence data.

[0046] When the obtained DNA encoding an amino acid sequence having homology to the amino acid sequence of human phospholipase A₂β (GenBank: AAC78836) is a partial nucleotide sequence, like EST (Expressed Sequence Tag), the full length cDNA can be obtained in the following manner, and the DNA of the present invention can be obtained from the cDNA.

[0047] The origin of the DNA of the present invention is not particularly limited, but it is preferably mammals, more preferably human, rat or mouse.

(1) Preparation of cDNA Library

[0048] For the preparation of a cDNA library, total RNA or mRNA is prepared from an appropriate cell or tissue.

[0049] The methods for preparing total RNA include the guanidine thiocyanate-caesium trifluoroacetate method [Methods in Enzymology, 154, 3 (1987)] and the acidic guanidine thiocyanate-phenol-chloroform (AGPC) method [Analytical Biochemistry, 162, 156 (1987); Experimental Medicine, 9, 1937 (1991)].

[0050] The methods for preparing mRNA as poly(A)⁺RNA from the total RNA include the oligo (dT) immobilized cellulose column method (Molecular Cloning, Second Edition) and the method using oligo dT latex.

[0051] It is also possible to prepare mRNA directly from a tissue or cell by using a kit such as Fast Track mRNA Isolation Kit (Invitrogen) or Quick Prep mRNA Purification Kit (Pharmacia).

[0052] It is preferred to use, as the cell or tissue, those used to construct the cDNA library containing EST or the like which has been found in a database, or cell lines derived from such tissue.

[0053] A cDNA library is prepared by an ordinary method using the obtained total RNA or mRNA.

[0054] The methods for preparing the cDNA library include the methods described in Molecular Cloning, Second Edition; Current Protocols in Molecular Biology; DNA Cloning 1: Core Techniques, A Practical Approach, Second Edi-

tion, Oxford University Press (1995), and methods using commercially available kits such as Superscript Plasmid System for cDNA Synthesis and Plasmid Cloning (Gibco BRL) and ZAP-cDNA Synthesis Kit (STRATAGENE).

[0055] The cloning vector for preparing the cDNA library may be any phage vectors, plasmid vectors, etc. insofar as they can be autonomously replicated in *Escherichia coli* K12.

[0056] Examples of suitable vectors include ZAP Express [STRATAGENE; Strategies, 5, 58 (1992)], pBluescript II SK(+) [Nucleic Acids Research, 17, 9494 (1989)], Lambda ZAP II (STRATAGENE), λ gt10, λ gt11 [DNA Cloning, A Practical Approach, 1, 49 (1985)], λ TriplEx (Clontech), λ ExCell (Pharmacia), pT7T318U (Pharmacia), pCD2 [Mol. Cell. Biol., 3, 280 (1983)], pUC18 [Gene, 33, 103 (1985)] and pAMo [J. Biol. Chem., 268, 22782-22787 (1993), also called pAMoPRC3Sc (Japanese Published Unexamined Patent Application No. 336963/93)].

[0057] As the host microorganism, any microorganism belonging to *Escherichia coli* can be used. Examples of suitable host microorganisms are *Escherichia coli* XL1-Blue MRF' [STRATAGENE; Strategies, 5, 81 (1992)], *Escherichia coli* C600 [Genetics, 39, 440 (1954)], *Escherichia coli* Y1088 [Science, 222, 778 (1983)], *Escherichia coli* Y1090 [Science, 222, 778 (1983)], *Escherichia coli* NM522 [J. Mol. Biol., 166, 1 (1983)], *Escherichia coli* K802 [J. Mol. Biol., 16, 118 (1966)], *Escherichia coli* JM105 [Gene, 38, 275 (1985)], *Escherichia coli* SOLRTM Strain [STRATAGENE] and *Escherichia coli* LE392 (Molecular Cloning, Second Edition).

[0058] In addition to cDNA libraries prepared by the above methods, commercially available cDNA libraries may also be utilized.

[0059] The commercially available cDNA libraries include cDNA libraries of organs derived from human, cow, mouse, rat, rabbit, etc. which are available from Clontech, Lifetech Oriental, etc.

(2)(i) Acquisition of the DNA of the Present Invention

[0060] From the cDNA library prepared in the above (1), a cDNA clone containing the DNA of the present invention or a part thereof can be selected by colony hybridization or plaque hybridization (Molecular Cloning, Second Edition) using an isotope- or fluorescence-labeled probe.

[0061] Useful probes include fragments obtained by amplifying a part of the cDNA by PCR [PCR Protocols, Academic Press (1990)] using primers based on a known partial nucleotide sequence, and oligonucleotides based on a known partial nucleotide sequence.

[0062] When the nucleotide sequences of both the 5' terminal and 3' terminal regions of the full length cDNA have been clarified by EST or the like, primers prepared based on the nucleotide sequences can be used.

[0063] An adapter is attached to the ends of the cDNA, and PCR is carried out using primers based on the nucleotide sequence of the adapter and the known partial sequence. By this procedure, i.e., 5'-RACE (rapid amplification of cDNA ends) and 3'-RACE [Proc. Natl. Acad. Sci. USA, 85, 8998 (1988)], cDNA fragments at the 5' side and 3' side of the sequence used for preparing the primers can be obtained.

[0064] By ligating the obtained cDNA fragments, the full length DNA of the present invention can be obtained.

[0065] When the cDNA obtained from the above cDNA library does not encode the full length polypeptide, the cDNA encoding the full length polypeptide can be obtained in the following manner.

[0066] PCR is carried out using, as templates, single stranded cDNA libraries prepared from various organs or cells or cDNA libraries prepared by the above methods, and as primers, a set of primers specific for the cDNA, whereby the organ or cell expressing the DNA corresponding to the cDNA can be specified. By subjecting the cDNA library derived from the specified organ or cell to colony hybridization (Molecular Cloning, Second Edition) using the cDNA as a probe, the cDNA containing the full length cDNA can be selected from the cDNA library.

[0067] The single stranded cDNA libraries derived from various organs or cells can be prepared according to conventional methods or by use of commercially available kits, for example, in the following manner.

[0068] Total RNA is extracted from various organs or cells by the guanidium thiocyanate-phenol-chloroform method [Anal. Biochem., 162, 156 (1987)] and then, if necessary, treated with deoxyribonuclease I (Life Technologies) to decompose contaminating chromosomal DNA. From each of the obtained total RNAs, a single stranded cDNA library can be prepared by SUPERScript™ Preamplification System for First Strand cDNA System (Life Technologies) using oligo (dT) primers or random primers.

[0069] The nucleotide sequence of the DNA obtained by the above method can be determined by inserting the DNA fragment, as such or after cleavage with appropriate restriction enzymes, into a vector by a conventional method, and then analyzing the sequence by generally employed methods such as the dideoxy method of Sanger, et al. [Proc. Natl. Acad. Sci. USA, 74, 5463 (1977)] or by use of nucleotide sequencers such as Perkin Elmer: 373A-DNA Sequencer and those available from Pharmacia, LI-COR, etc.

[0070] A specific example of a plasmid containing the DNA of the present invention obtained by the above method is plasmid p5269+C5 comprising the DNA consisting of the nucleotide sequence shown in SEQ ID NO: 2.

[0071] *Escherichia coli* JM109/p5269+C5 carrying plasmid p5269+C5 was deposited with International Patent Organism Depository, National Institute of Advanced Industrial Science and Technology, Central 6, 1-1, Higashi 1-chome,

Tsukuba-shi, Ibaraki, Japan (former name: National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki, Japan), on August 25, 2000 with accession No. FERM BP-7281.

[0072] By selecting DNA which hybridizes under stringent conditions to the DNA obtained by the above method, desired DNA derived from other tissues or animals (e.g., human and mouse) can be obtained.

[0073] The desired DNA can also be prepared by chemical synthesis using a DNA synthesizer on the basis of the nucleotide sequence information obtained by the above method. Useful DNA synthesizers include the one utilizing the thiophosphite method (Shimadzu Corporation) and the one utilizing the phosphoamidite method (Model 392, Perkin Elmer).

[0074] The novelty of the obtained nucleotide sequence can be confirmed by search of nucleotide sequence databases such as GenBank, EMBL and DDBJ using a homology search program such as BLAST.

[0075] The novel nucleotide sequence may be converted to an amino acid sequence, and the obtained amino acid sequence can be used for search of amino acid sequence databases such as GenPept, PIR and Swiss-Prot using a homology search program such as FASTA or FrameSearch for DNA having homology.

(ii) Acquisition of DNA Encoding the Inhibitor Polypeptide

[0076] The DNA encoding the inhibitor polypeptide can be obtained by deleting the region considered to be the active domain from the DNA of the present invention obtained in the above (2)-(i) by a known method, for example, the method described in Molecular Cloning, Second Edition.

(3) Preparation of the Oligonucleotides of the Present Invention

[0077] Oligonucleotides such as antisense oligonucleotides and sense oligonucleotides having a partial sequence of the DNA of the present invention can be prepared according to a conventional method or by use of the above-mentioned DNA synthesizer using the DNA or DNA fragment of the present invention obtained by the above method.

[0078] Such oligonucleotides include DNA having a nucleotide sequence identical with a sequence of 5 to 60 consecutive nucleotides in the nucleotide sequence of the above DNA, and DNA having a nucleotide sequence complementary to that of said DNA. Examples of such oligonucleotides include DNA having a nucleotide sequence identical with a sequence of 5 to 60 consecutive nucleotides in the nucleotide sequence selected from the group consisting of the nucleotide sequences shown in SEQ ID NOS: 2, 23, 27 and 39, and DNA having a nucleotide sequence complementary to that of said DNA. When the above oligonucleotides are used as sense and antisense primers, it is preferred to use those of which the melting temperatures (T_m) and numbers of nucleotides are not markedly different from each other.

[0079] Specific examples of the oligonucleotides are the oligonucleotides shown in SEQ ID NOS: 13, 14, 28, 29, 30, 31, 46 and 47.

[0080] Further, derivatives of these oligonucleotides (also referred to hereinafter as oligonucleotide derivatives) can also be used as the oligonucleotides of the present invention.

[0081] The oligonucleotide derivatives include an oligonucleotide derivative wherein the phosphodiester bond in an oligonucleotide is converted to a phosphorothioate bond, an oligonucleotide derivative wherein the phosphodiester bond in an oligonucleotide is converted to an N3'-P5' phosphoamidate bond, an oligonucleotide derivative wherein the ribose-phosphodiester bond in an oligonucleotide is converted to a peptide-nucleic acid bond, an oligonucleotide derivative wherein the uracil in an oligonucleotide is substituted by C-5 propynyluracil, an oligonucleotide derivative wherein the uracil in an oligonucleotide is substituted by C-5 thiazolyluracil, an oligonucleotide derivative wherein the cytosine in an oligonucleotide is substituted by C-5 propynylcytosine, an oligonucleotide derivative wherein the cytosine in an oligonucleotide is substituted by phenoxazine-modified cytosine, an oligonucleotide derivative wherein the ribose in an oligonucleotide is substituted by 2'-O-propylribose, and an oligonucleotide derivative wherein the ribose in an oligonucleotide is substituted by 2'-methoxyethoxyribose [Cell Technology, 16, 1463 (1997)].

[2] Preparation of the Polypeptide and Inhibitor

Polypeptide of the Present Invention

(1) Preparation of a Transformant

[0082] The polypeptide or inhibitor polypeptide of the present invention can be produced by expressing the DNA of the present invention or the DNA encoding the inhibitor polypeptide obtained by the methods described in the above [1] in host cells using the methods described in Molecular Cloning, Second Edition, Current Protocols in Molecular

Biology, etc.

[0083] That is, the polypeptide or inhibitor polypeptide of the present invention can be produced by inserting the DNA of the present invention or the DNA encoding the inhibitor polypeptide into an appropriate expression vector at an insertion site located downstream of the promoter therein to construct a recombinant vector, introducing the recombinant vector into a host cell to obtain a transformant expressing the polypeptide or inhibitor polypeptide of the present invention, and culturing the transformant.

[0084] As the host cell, any microorganisms (e.g., bacteria and yeast), animal cells, insect cells, plant cells, etc. that are capable of expressing the desired DNA can be used.

[0085] The expression vectors that can be employed are those capable of autonomous replication or integration into the chromosome in the above host cells and comprising a promoter at a position appropriate for the transcription of the DNA of the present invention or the DNA encoding the inhibitor polypeptide.

[0086] When a procaryote such as a bacterium is used as the host cell, it is preferred that the recombinant vector comprising the DNA of the present invention or the DNA encoding the inhibitor polypeptide is a recombinant vector which is capable of autonomous replication in the procaryote and which comprises a promoter, a ribosome binding sequence, the DNA of the present invention or the DNA encoding the inhibitor polypeptide, and a transcription termination sequence. The vector may further comprise a DNA regulating the promoter.

[0087] Examples of suitable expression vectors are pBTrp2, pBTac1 and pBTac2 (all commercially available from Boehringer Mannheim), pKK233-2 (Pharmacia), pSE280 (Invitrogen), pGEMEX-1 (Promega), pQE-8 (QIAGEN), pKYP10 (Japanese Published Unexamined Patent Application No. 110600/83), pKYP200 [Agric. Biol. Chem., 48, 669 (1984)], pLSA1 [Agric. Biol. Chem., 53, 277 (1989)], pGEL1 [Proc. Natl. Acad. Sci. USA, 82, 4306 (1985)], pBluescript II SK(-) (STRATAGENE), pTrs32 (FERM BP-5408), pGHA2 (FERM BP-400), pGKA2 (FERM BP-6798), pTerm2 (Japanese Published Unexamined Patent Application No. 22979/91, US4686191, US4939094, US5160735), pGEX (Pharmacia), pET-3 (Novagen), pSupex, pUB110, pTP5, pC194, pTrxFus (Invitrogen), and pMAL-c2 (New England Biolabs).

[0088] As the promoter, any promoters capable of functioning in host cells such as Escherichia coli and Bacillus subtilis can be used. For example, promoters derived from Escherichia coli or phage, such as trp promoter (P_{trp}), lac promoter (P_{lac}), P_L promoter, P_R promoter and T7 promoter, SPO1 promoter, SPO2 promoter and penP promoter can be used. Artificially designed and modified promoters such as a promoter in which two P_{trps} are combined in tandem (P_{trp} x 2), tac promoter, lacT7 promoter and letI promoter, etc. can also be used.

[0089] It is preferred to use a plasmid in which the distance between the Shine-Dalgarno sequence (ribosome binding sequence) and the initiation codon is adjusted to an appropriate length (e.g., 6 to 18 bases).

[0090] Although a transcription termination sequence is not essential for the expression of the DNA of the present invention or the DNA encoding the inhibitor polypeptide, it is preferred to place the transcription termination sequence immediately downstream of the structural gene.

[0091] Examples of suitable host cells are microorganisms belonging to the genera Escherichia, Serratia, Bacillus, Brevibacterium, Corynebacterium, Microbacterium and Pseudomonas, specifically, Escherichia coli XL1-Blue, Escherichia coli XL2-Blue, Escherichia coli DH1, Escherichia coli MC1000, Escherichia coli KY3276, Escherichia coli W1485, Escherichia coli JM109, Escherichia coli HB101, Escherichia coli No. 49, Escherichia coli W3110, Escherichia coli NY49, Serratia ficaria, Serratia fonticola, Serratia liquefaciens, Serratia marcescens, Bacillus subtilis, Bacillus amyloliquefaciens, Brevibacterium ammoniagenes, Brevibacterium immariophilum ATCC 14068, Brevibacterium saccharolyticum ATCC 14066, Corynebacterium glutamicum ATCC 13032, Corynebacterium glutamicum ATCC 14067, Corynebacterium glutamicum ATCC 13869, Corynebacterium acetoacidophilum ATCC 13870, Microbacterium ammoniophilum ATCC 15354 and Pseudomonas sp. D-0110.

[0092] Introduction of the recombinant vector can be carried out by any of the methods for introducing DNA into the above host cells, for example, the method using calcium ion [Proc. Natl. Acad. Sci. USA, 69, 2110 (1972)], the protoplast method (Japanese Published Unexamined Patent Application No. 248394/88) and electroporation [Gene, 17, 107 (1982); Molecular & General Genetics, 168, 111 (1979)].

[0093] When yeast is used as the host cell, YEp13 (ATCC 37115), YEp24 (ATCC 37051), YCp50 (ATCC 37419), pHS19, pHS15, etc. can be used as the expression vector.

[0094] As the promoter, any promoters capable of functioning in yeast can be used. Suitable promoters include PH05 promoter, PGK promoter, GAP promoter, ADH promoter, gal 1 promoter, gal 10 promoter, heat shock polypeptide promoter, MF α 1 promoter, CUP1 promoter, etc.

[0095] Examples of suitable host cells are yeast strains belonging to the genera Saccharomyces, Schizosaccharomyces, Kluyveromyces, Trichosporon and Schwanniomyces, specifically, Saccharomyces cerevisiae, Schizosaccharomyces pombe, Kluyveromyces lactis, Trichosporon pullulans, Schwanniomyces alluvius and Pichia pastoris.

[0096] Introduction of the recombinant vector can be carried out by any of the methods for introducing DNA into yeast, for example, the electroporation method [Methods in Enzymology, 194, 182 (1990)], the spheroplast method [Proc. Natl. Acad. Sci. USA, 81, 4889 (1984)] and the lithium acetate method [Journal of Bacteriology, 153, 163 (1983)].

[0097] When an animal cell is used as the host cell, pcDNAI/Amp (Invitrogen), pcDNAI, pCDM8 [Nature, 329, 840

(1987)], pAGE107 [Japanese Published Unexamined Patent Application No. 22979/91; Cytotechnology, 3, 133 (1990)], pREP4 (Invitrogen), pAGE103 [Journal of Biochemistry, 101, 1307 (1987)], pAMo, pAMoA, pAS3-3 (Japanese Published Unexamined Patent Application No. 227075/90), etc. can be used as the expression vector.

[0098] As the promoter, any promoters capable of functioning in animal cells can be used. Suitable promoters include the promoter of IE (immediate early) gene of cytomegalovirus (CMV), SV40 early promoter, metallothionein promoter, the promoter of a retrovirus, heat shock promoter, SR α promoter, etc. The enhancer of IE gene of human CMV may be used in combination with the promoter.

[0099] Examples of suitable animal cells are mouse myeloma cells, rat myeloma cells, mouse hybridomas, human-derived Namalwa cells and Namalwa KJM-1 cells [Cytotechnology, 1, 151 (1988)], human embryonic kidney cells, human leukemia cells, African green monkey kidney cells, Chinese hamster-derived CHO cells, and HBT5637 (Japanese Published Unexamined Patent Application No. 000299/88).

[0100] The mouse myeloma cells include SP2/0 and NSO; the rat myeloma cells include YB2/0; the human embryonic kidney cells include HEK293 (ATCC: CRL-1573); the human leukemia cells include BALL-1; and the African green monkey kidney cells include COS-1 and COS-7.

[0101] Introduction of the recombinant vector can be carried out by any of the methods for introducing DNA into animal cells, for example, the electroporation method [Cytotechnology, 3, 133 (1990)], the calcium phosphate method (Japanese Published Unexamined Patent Application No. 227075/90), the lipofection method [Proc. Natl. Acad. Sci. USA, 84, 7413 (1987)], and the method described in Virology, 52, 456 (1973).

[0102] When an insect cell is used as the host cell, the polypeptide can be expressed by using the methods described in Baculovirus Expression Vectors, A Laboratory Manual, W. H. Freeman and Company, New York (1992); Molecular Biology, A Laboratory Manual; Current Protocols in Molecular Biology; Bio/Technology, 6, 47 (1988), etc.

[0103] That is, the recombinant DNA transfer vector and a baculovirus are cotransfected into insect cells to obtain a recombinant virus in the culture supernatant of the insect cells, and then insect cells are infected with the recombinant virus, whereby the polypeptide can be expressed.

[0104] The DNA transfer vectors useful in this method include pVL1392, pVL1393 and pBlueBacIII (products of Invitrogen).

[0105] An example of the baculovirus is Autographa californica nuclear polyhedrosis virus, which is a virus infecting insects belonging to the family Barathra.

[0106] Examples of the insect cells are ovarian cells of Spodoptera frugiperda, ovarian cells of Trichoplusia ni, and cultured cells derived from silkworm ovary.

[0107] The ovarian cells of Spodoptera frugiperda include Sf9 and Sf21 (Baculovirus Expression Vectors, A Laboratory Manual); the ovarian cells of Trichoplusia ni include High 5 and BTI-TN-5B1-4 (Invitrogen); and the cultured cells derived from silkworm ovary include Bombyx mori N4.

[0108] Cotransfection of the above recombinant DNA transfer vector and the above baculovirus into insect cells for the preparation of the recombinant virus can be carried out by the calcium phosphate method (Japanese Published Unexamined Patent Application No. 227075/90), lipofection [Proc. Natl. Acad. Sci. USA, 84, 7413 (1987)], etc.

[0109] Expression of the DNA can be carried out not only by direct expression but also by secretory production, fused protein expression, etc. according to the methods described in Molecular Cloning, Second Edition, etc.

[0110] When the DNA is expressed in yeast, an animal cell or an insect cell, a glycosylated polypeptide can be obtained.

[0111] The polypeptide or inhibitor polypeptide of the present invention can be produced by culturing the transformant obtained as above in a medium, allowing the polypeptide or inhibitor polypeptide of the present invention to form and accumulate in the culture, and recovering the polypeptide or inhibitor polypeptide from the culture.

[0112] The polypeptide or inhibitor polypeptide of the present invention can also be expressed in the body of a patient by introducing an appropriate recombinant vector for the expression of the polypeptide or inhibitor polypeptide of the present invention into cells collected from the body of the patient and then returning the cells into the body of the patient.

(2) Culturing of the Transformant

[0113] Culturing of the transformant of the present invention in a medium can be carried out by conventional methods for culturing the host of the transformant.

[0114] For the culturing of the transformant prepared by using a procaryote such as Escherichia coli or a eucaryote such as yeast as the host, any of natural media and synthetic media can be used insofar as it is a medium suitable for efficient culturing of the transformant which contains carbon sources, nitrogen sources, inorganic salts, etc. which can be assimilated by the host used.

[0115] As the carbon sources, any carbon sources that can be assimilated by the host can be used. Examples of suitable carbon sources include carbohydrates such as glucose, fructose, sucrose, molasses containing them, starch and starch hydrolyzate; organic acids such as acetic acid and propionic acid; and alcohols such as ethanol and pro-

panol.

[0116] As the nitrogen sources, ammonia, ammonium salts of organic or inorganic acids such as ammonium chloride, ammonium sulfate, ammonium acetate and ammonium phosphate, and other nitrogen-containing compounds can be used as well as peptone, meat extract, yeast extract, corn steep liquor, casein hydrolyzate, soybean cake, soybean cake hydrolyzate, and various microbial cells obtained by fermentation and digested products thereof.

[0117] Examples of the inorganic salts include potassium dihydrogenphosphate, dipotassium hydrogenphosphate, magnesium phosphate, magnesium sulfate, sodium chloride, ferrous sulfate, manganese sulfate, copper sulfate and calcium carbonate.

[0118] Culturing is usually carried out under aerobic conditions, for example, by shaking culture or submerged spinner culture under aeration. The culturing temperature is preferably 15 to 40°C, and the culturing period is usually 16 to 96 hours. The pH is maintained at 3.0 to 9.0 during the culturing. The pH adjustment is carried out by using an organic or inorganic acid, an alkali solution, urea, calcium carbonate, ammonia, etc.

[0119] If necessary, antibiotics such as ampicillin and tetracycline may be added to the medium during the culturing.

[0120] When a microorganism transformed with a recombinant vector comprising an inducible promoter is cultured, an inducer may be added to the medium, if necessary. For example, in the case of a microorganism transformed with a recombinant vector comprising lac promoter, isopropyl-β-D-thiogalactopyranoside or the like may be added to the medium; and in the case of a microorganism transformed with a recombinant vector comprising trp promoter, indole-acrylic acid or the like may be added.

[0121] For the culturing of the transformant prepared by using an animal cell as the host cell, generally employed media such as RPMI1640 medium [The Journal of the American Medical Association, 199, 519 (1967)], Eagle's MEM [Science, 122, 501 (1952)], DMEM [Virology, 8, 396 (1959)] and 199 medium [Proceeding of the Society for the Biological Medicine, 73, 1 (1950)], media prepared by adding fetal calf serum or the like to these media, etc. can be used as the medium.

[0122] Culturing is usually carried out at pH 6 to 8 at 30 to 40°C for 1 to 7 days in the presence of 5% CO₂.

[0123] If necessary, antibiotics such as kanamycin, penicillin and streptomycin may be added to the medium during the culturing.

[0124] For the culturing of the transformant prepared by using an insect cell as the host cell, generally employed media such as TNM-FH medium (PharMingen), Sf-900II SFM medium (Life Technologies), ExCell 400 and ExCell 405 (JRH Biosciences) and Grace's Insect Medium [Nature, 195, 788 (1962)] can be used as the medium.

[0125] Culturing is usually carried out at pH 6 to 7 at 25 to 30°C for 1 to 5 days.

[0126] If necessary, antibiotics such as gentamicin may be added to the medium during the culturing.

(3) Isolation and Purification of the Expressed Polypeptide

[0127] The polypeptide expressed by the above method can be isolated and purified from a culture of the above transformant by conventional methods for isolating and purifying enzymes.

[0128] For example, when the polypeptide or inhibitor polypeptide of the present invention is expressed in a soluble form in cells, the cells are recovered by centrifugation after the completion of culturing and suspended in an aqueous buffer, followed by disruption using a sonicator, French press, Manton Gaulin homogenizer, Dynamill or the like to obtain a cell-free extract.

[0129] A purified polypeptide preparation can be obtained by centrifuging the cell-free extract to obtain the supernatant and then subjecting the supernatant to ordinary means for isolating and purifying enzymes, e.g., extraction with a solvent, salting-out with ammonium sulfate, etc., desalting, precipitation with an organic solvent, anion exchange chromatography using resins such as diethylaminoethyl (DEAE)-Sephacrose and DIAION HPA-75 (Mitsubishi Chemical Corporation), cation exchange chromatography using resins such as S-Sepharose FF (Pharmacia), hydrophobic chromatography using resins such as butyl Sepharose and phenyl Sepharose, gel filtration using a molecular sieve, affinity chromatography, chromatofocusing, and electrophoresis such as isoelectric focusing, alone or in combination.

[0130] When the polypeptide is expressed as an inclusion body in cells, the cells are similarly recovered and disrupted, followed by centrifugation to obtain a precipitate fraction. After the polypeptide is recovered from the precipitate fraction by an ordinary method, the inclusion body of the polypeptide is solubilized with a protein-denaturing agent.

[0131] The solubilized polypeptide solution is diluted with or dialyzed against a solution containing no protein-denaturing agent or a solution containing the protein-denaturing agent at such a low concentration that denaturation of protein is not caused, whereby the polypeptide is renatured to have normal higher-order structure. Then, a purified polypeptide preparation can be obtained by the same isolation and purification steps as described above.

[0132] When the polypeptide or inhibitor polypeptide of the present invention or its derivative such as a glycosylated form is extracellularly secreted, the polypeptide or its derivative such as a glycosylated form can be recovered in the culture supernatant.

[0133] That is, the culture is treated by the same means as above, e.g., centrifugation, to obtain a soluble fraction.

A purified polypeptide preparation can be obtained from the soluble fraction by using the same isolation and purification methods as described above.

[0134] It is also possible to produce the polypeptide or inhibitor polypeptide of the present invention as a fusion protein with another protein and to purify it by affinity chromatography using a substance having affinity for the fused protein. For example, according to the method of Lowe, et al. [Proc. Natl. Acad. Sci. USA, **86**, 8227 (1989); Genes & Dev., **4**, 1288 (1990)] and the methods described in Japanese Published Unexamined Patent Application No. 336963/93 and WO94/23021, the polypeptide or inhibitor polypeptide of the present invention can be produced as a fusion protein with protein A and can be purified by affinity chromatography using immunoglobulin G. Further, it is possible to produce the polypeptide or inhibitor polypeptide of the present invention as a fusion protein with a Flag peptide and to purify it by affinity chromatography using anti-Flag antibody [Proc. Natl. Acad. Sci. USA, **86**, 8227 (1989); Genes & Dev., **4**, 1288 (1990)]. The polypeptide can also be purified by affinity chromatography using an antibody against said polypeptide.

[0135] The polypeptide or inhibitor polypeptide of the present invention can also be produced by chemical synthetic methods such as the Fmoc method (the fluorenylmethyloxycarbonyl method) and the tBoc method (the t-butyloxycarbonyl method).

[0136] Further, the polypeptide or inhibitor polypeptide of the present invention can be chemically synthesized by using peptide synthesizers from Advanced ChemTech, Perkin Elmer, Pharmacia, Protein Technology Instrument, Synthecell-Vega, PerSeptive, Shimadzu Corporation, etc.

[0137] The structural analysis of the purified polypeptide or inhibitor polypeptide of the present invention can be carried out according to methods generally employed in protein chemistry, e.g., the method described in Structural Analysis of Protein for DNA Cloning (Hisashi Hirano, Tokyo Kagaku Dojin, 1993).

[3] Preparation of an Antibody Recognizing the Polypeptide of the Present Invention

(1) Preparation of a Polyclonal Antibody

[0138] A polyclonal antibody can be prepared by using, as an antigen, a purified preparation of the full length polypeptide of the present invention or a partial fragment thereof obtained by the method described in [2] above and administering the antigen to an animal.

[0139] The animals to which the antigen is administered include rabbits, goats, rats, mice and hamsters.

[0140] The dose of the antigen is preferably 50 to 100 µg per animal.

[0141] When a peptide is used as the antigen, it is preferred to use the peptide as the antigen after binding it covalently to a carrier protein such as keyhole limpet haemocyanin or bovine thyroglobulin. The peptide used as the antigen can be synthesized by a peptide synthesizer.

[0142] Administration of the antigen is carried out 3 to 10 times at one- to two-week intervals after the first administration. A blood sample is collected from the fundus oculi veniplex on the third to seventh day after each administration, and the serum is examined for reactivity to the antigen used for immunization by enzyme immunoassay [Enzyme-linked Immunosorbent Assay (ELISA), published by Igaku Shoin (1976); Antibodies - A Laboratory Manual, Cold Spring Harbor Laboratory Press (1988)] or the like.

[0143] The polyclonal antibody can be prepared by obtaining serum from a non-human mammal whose serum shows a sufficient antibody titer against the antigen used for immunization, and separating and purifying it from the serum.

[0144] Separation and purification of the polyclonal antibody can be carried out by centrifugation, salting-out with 40 to 50% saturated ammonium sulfate, caprylic acid precipitation [Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory (1988)], chromatography using a DEAE-Sepharose column, an anion exchange column, a protein A or G column or a gel filtration column, and the like, alone or in combination.

(2) Preparation of a Monoclonal Antibody

(2-1) Preparation of Antibody-producing Cells

[0145] A rat whose serum shows a sufficient antibody titer against the antigen used for immunization in (1) above is used as a source of antibody-producing cells.

[0146] On the third to seventh day after the final administration of the antigen to the rat showing such antibody titer, the spleen is excised from the rat.

[0147] The spleen is cut into small pieces in MEM (Nissui Pharmaceutical Co., Ltd.) and the pieces are loosened with tweezers, followed by centrifugation at 1,200 rpm for 5 minutes. The resulting supernatant is discarded.

[0148] The spleen cells in the obtained precipitate fraction are treated with a Tris-ammonium chloride buffer (pH 7.65) for 1 to 2 minutes to remove erythrocytes, and then washed three times with MEM to give spleen cells to be used

as antibody-producing cells.

(2-2) Preparation of Myeloma Cells

[0149] As the myeloma cells, cell lines obtained from mouse or rat are used.

[0150] Examples of suitable cell lines are 8-azaguanine-resistant mouse (BALB/c-derived) myeloma cell line P3-X63Ag8-U1 (P3-U1) [Curr. Topics Microbiol. Immunol., 81, 1 (1978); Eur. J. Immunol., 6, 511 (1976)], SP2/0-Ag14 (SP-2) [Nature, 276, 269 (1978)], P3-X63-Ag8653 (653) [J. Immunol., 123, 1548 (1979)] and P3-X63-Ag8 (X63) [Nature, 256, 495 (1975)]. These cell lines are subcultured in 8-azaguanine medium [medium prepared by adding 8-azaguanine (15 µg/ml) to a medium (referred to hereinafter as normal medium) prepared by adding glutamine (1.5 mmol/l), 2-mercaptoethanol (5×10^{-5} mol/l), gentamicin (10 µg/ml) and fetal calf serum (FCS) (a product of CSL Ltd.; 10%) to RPMI-1640 medium], and 3 to 4 days before cell fusion, they are cultured in the normal medium. At least 2×10^7 cells are used for the fusion.

(2-3) Preparation of Hybridoma

[0151] The antibody-producing cells obtained in (2-1) and the myeloma cells obtained in (2-2) are washed well with MEM or PBS (1.83 g of disodium phosphate, 0.21 g of monopotassium phosphate, 7.65 g of sodium chloride, 1 L of distilled water, pH 7.2) and mixed at the antibody-producing cells/myeloma cells ratio of 5/1 to 10/1. The mixture is centrifuged at 1,200 rpm for 5 minutes, and the supernatant is discarded.

[0152] The cells in the precipitate fraction are loosened well, and a mixture of 2 g of polyethylene glycol-1000 (PEG-1000), 2 ml of MEM and 0.7 ml of dimethyl sulfoxide (DMSO) is added to the cells in an amount of 0.2 to 1 ml per 10^8 antibody-producing cells with stirring at 37°C. Then, 1 to 2 ml of MEM is added thereto several times at 1- to 2-minute intervals.

[0153] After the addition, MEM is further added to adjust the total volume to 50 ml.

[0154] The mixture thus prepared is centrifuged at 900 rpm for 5 minutes, and the supernatant is discarded.

[0155] The cells in the obtained precipitate fraction are gently loosened and then suspended in 100 ml of HAT medium [medium prepared by adding hypoxanthine (10^{-4} mol/l), thymidine (1.5×10^{-5} mol/l) and aminopterin (4×10^{-7} mol/l) to the normal medium] by gentle pipetting using a measuring pipette.

[0156] The resulting suspension is put into wells of a 96-well culture plate in an amount of 100 µl/well, and cultured in a 5% CO₂ incubator at 37°C for 7 to 14 days.

[0157] After the culturing, an aliquot of the culture supernatant is sampled and subjected to enzyme immunoassay described in Antibodies - A Laboratory Manual, Cold Spring Harbor Laboratory Press, Chapter 14 (1988) or the like to select a hybridoma specifically reacting with the polypeptide of the present invention.

[0158] Enzyme immunoassay can be carried out, for example, in the following manner.

[0159] An appropriate plate is coated with the purified preparation of the full length polypeptide of the present invention or a partial fragment thereof used as the antigen for immunization, followed by reaction with a culture supernatant of the hybridoma or the purified antibody obtained in (2-4) below as a first antibody and then with anti-rat immunoglobulin antibody labeled with biotin, an enzyme, a chemiluminescent substance or a radioisotope as a second antibody. Then, reaction according to the labeling substance is conducted, and hybridomas specifically reacting with the polypeptide of the present invention are selected as hybridomas producing a monoclonal antibody against the polypeptide of the present invention.

[0160] Using the obtained hybridomas, cloning is carried out twice by limiting dilution [first cloning: HT medium (a medium having the composition of HAT medium excluding aminopterin) is used, second cloning: the normal medium is used]. A hybridoma showing a high and stable antibody titer is selected as the hybridoma strain producing a monoclonal antibody against the polypeptide of the present invention.

(2-4) Preparation of a Monoclonal Antibody

[0161] The hybridoma cells producing a monoclonal antibody against the polypeptide of the present invention, obtained in (2-3), are intraperitoneally injected into 8 to 10-week-old mice or nude mice treated with Pristane [animals raised for 2 weeks after intraperitoneal administration of 0.5 ml of 2,6,10,14-tetramethylpentadecane (Pristane)] in an amount of 5 to 20×10^6 cells/animal. The hybridoma forms ascites tumor in 10 to 21 days.

[0162] The ascites is collected from the mouse with ascites tumor and centrifuged at 3,000 rpm for 5 minutes to remove the solid matters.

[0163] From the resulting supernatant, the monoclonal antibody can be purified and obtained according to the same method as used for obtaining the polyclonal antibody.

[0164] The subclass of the antibody is determined using a mouse monoclonal antibody typing kit or a rat monoclonal

antibody typing kit. The amount of the polypeptide is calculated by the Lowry method or from the absorbance at 280 nm.

[4] Measurement of the Phospholipase A₂ Activity of the Polypeptide of the Present Invention

[0165] The polypeptide of the present invention expressed in hosts such as *Escherichia coli*, yeast, insect cells and animal cells by the methods described in [2] above, the polypeptide expressed in oocytes of *Xenopus* by microinjection [Methods in Enzymology, 207, 225 (1992); Methods in Enzymology, 254, 458 (1995)] using DNA or cRNA prepared *in vitro*, the polypeptide produced by *in vitro* translation, etc. are subjected to measurement of phospholipase A₂ activity. The phospholipase A₂ activity is measured by quantitatively determining a hydrolyzate (e.g., [1-¹⁴C] arachidonic acid) of a substrate (e.g., 1-palmitoyl-2-[1-¹⁴C] arachidonyl-phosphatidylcholine) labeled with a detectable reagent (e.g., a radioactive reagent, a fluorescent reagent or a colorimetric reagent) or a remaining substrate. The phospholipase A₂ activity can also be measured by quantitatively determining an unlabeled substrate or a decomposition product [Methods in Enzymology, 197, 3 (1991)].

[5] Search for and Identification of an Agonist or Antagonist of the Polypeptide of the Present Invention and Utilization Thereof as a Therapeutic Agent

[0166] A test sample is added to a sample containing cells useful in the measurement of activity described in [4] above or tissue or cells confirmed to express the polypeptide of the present invention or its mRNA by the method described in [7] below, followed by measurement of phospholipase A₂ activity according to the method described in [4] above.

[0167] The sample may be in any form so far as the tissue or cells can exhibit phospholipase A₂ activity.

[0168] Substances enhancing phospholipase A₂ activity (agonists) and substances inhibiting phospholipase A₂ activity (antagonists) can be identified by screening of test samples based on the comparison of the phospholipase A₂ activity of the polypeptide of the present invention in the presence and absence of a test sample.

[0169] Suitable test samples include synthetic compounds, proteins existing in nature, artificially synthesized proteins, peptides, glucides, lipids, and modified forms or derivatives thereof; urine, body fluids, tissue extracts, culture supernatant of cells, and cell extracts derived from mammals (e.g., mouse, rat, guinea pig, hamster, pig, sheep, cow, horse, dog, cat, monkey and human); and nonpeptide compounds, fermentation products, and extracts of plants or other organisms.

[0170] The agonist or antagonist of the polypeptide of the present invention obtained by the above method may be used alone as a therapeutic agent. However, it is preferably mixed with one or more pharmaceutically acceptable carrier and used as a pharmaceutical preparation produced by any of the methods well known in the technical field of pharmaceuticals.

[0171] The agonist can be used as an ingredient of a preventing or therapeutic agent for diabetes.

[0172] The antagonist can be used as an ingredient of a preventing or therapeutic agent for diabetes and other diseases such as asthma, ischemic diseases, arthritis, rheumatism, sepsis, dermatitis, arteriosclerosis, pain, Parkinson disease, Alzheimer disease, malignant tumor, nephritis and ischemic reperfusion injury. The antagonist includes the inhibitor polypeptide.

[0173] It is desirable to administer the therapeutic agent by the route that is most effective for the treatment. Suitable administration routes include oral administration and parenteral administration such as intra-oral cavity administration, intratracheal administration, intrarectal administration, subcutaneous administration, intramuscular administration and intravenous administration.

[0174] The therapeutic agent may be in the form of ointment, spray, capsules, tablets, granules, syrup, emulsion, suppository, injection, tape, and the like.

[0175] The pharmaceutical preparations suitable for oral administration include emulsions, syrups, capsules, tablets, powders and granules.

[0176] Liquid preparations such as emulsions and syrups can be prepared using, as additives, water, sugars (e.g., sucrose, sorbitol and fructose), glycols (e.g., polyethylene glycol and propylene glycol), oils (e.g., sesame oil, olive oil and soybean oil), antiseptics (e.g., p-hydroxybenzoates), flavors (e.g., strawberry flavor and peppermint), and the like.

[0177] Capsules, tablets, powders, granules, etc. can be prepared using, as additives, excipients (e.g., lactose, glucose, sucrose and mannitol), disintegrators (e.g., starch and sodium alginate), lubricants (e.g., magnesium stearate and talc), binders (e.g., polyvinyl alcohol, hydroxypropyl cellulose and gelatin), surfactants (e.g., fatty acid esters), plasticizers (e.g., glycerin), and the like.

[0178] The pharmaceutical preparations suitable for parenteral administration include injections, suppositories and sprays.

[0179] Injections can be prepared using, for example, carriers comprising a salt solution, a glucose solution, or a mixture thereof.

[0180] Suppositories can be prepared using, for example, carriers such as cacao butter, hydrogenated fat and carboxylic acid.

[0181] The agonist or antagonist obtained above may be used as such in the form of spray. However, sprays are preferably prepared using carriers for dispersing said compound as fine particles to facilitate absorption thereof without stimulating the oral cavity or tracheal mucous membrane of a recipient.

[0182] Suitable carriers include lactose and glycerin.

[0183] It is also possible to prepare aerosols, dry powders, etc. according to the properties of the agonist or antagonist obtained above and the carriers used.

[0184] In preparing these parenteral preparations, the above-mentioned additives for the oral preparations may also be added.

[0185] The dose and administration schedule will vary depending on the desired therapeutic effect, the administration route, the period of treatment, the patient's age and body weight, etc. However, an appropriate daily dose for an adult person is generally 10 µg/kg to 8 mg/kg. A similar dose is employed in the case of administration to non-human mammals.

[6] Search for and Identification of a Compound Regulating the Expression of the Polypeptide of the Present Invention (Hereinafter Referred to as Expression-regulating Compound)

(1) Search for and Identification of an Expression-regulating Compound Using the Antibody of the Present Invention

[0186] A compound regulating the expression of the polypeptide of the present invention can be searched for and identified by using the antibody of the present invention after contacting a test sample with cells expressing the polypeptide of the present invention.

[0187] The cells may be any cells, cell lines or tissues expressing the polypeptide of the present invention.

[0188] For example, cells, cell lines or tissues confirmed to express the polypeptide by the immunological detection method using antibodies described in [7] below can be used.

[0189] Preferred cell lines include those derived from kidney.

[0190] As the test sample, the test samples mentioned in [5] above can be used.

[0191] The cells expressing the polypeptide of the present invention are suspended in a medium allowing the growth of the cells, and a test sample is added to the medium for the contact with the cells. Then, the content of the polypeptide expressed in the cells is determined by using the antibody of the present invention. The determination can be carried out, for example, by the method utilizing immunocytochemical staining described below.

[0192] Cultured adherent cells are washed with PBS buffer, and 3 ml of PBS buffer containing 0.05% trypsin and 0.02% EDTA (ethylenediaminetetraacetic acid) is added thereto. After the removal of excess solution, incubation is carried out at 37°C for 5 minutes to detach the cells from the flask.

[0193] In the case of suspending cells, cultured cells can be used as such. After washing with PBS buffer, the cells are suspended in a fixative (e.g., PBS buffer containing 3.7% formaldehyde), followed by incubation at room temperature for 30 minutes. Then, the cells are washed with PBS buffer and suspended in a membrane-permeable reaction solution (e.g., PBS buffer containing 0.1% Triton X-100).

[0194] The cells thus treated are suspended in a buffer for immunocytochemical staining (e.g., PBS containing 1% BSA, 0.02% EDTA and 0.05% sodium azide) and put into wells of a 96-well round-bottom plate in an amount of 1 to 20 x 10⁵ cells/well.

[0195] To the wells of the above plate is added the monoclonal antibody of the present invention.

[0196] The monoclonal antibody may be a culture supernatant of the hybridoma producing the monoclonal antibody of the present invention obtained in [3] (2-3) above or the purified monoclonal antibody obtained in [3] (2-4) above. Also useful is an antibody prepared by labeling said monoclonal antibody.

[0197] An example of the antibody prepared by labeling said monoclonal antibody is a biotin-labeled antibody.

[0198] The biotin-labeled antibody can be prepared by a known method (Enzyme Antibody Technique, published by Gakusai Kikaku, 1985).

[0199] The above antibody is diluted with a buffer for immunocytochemical staining or a buffer for immunocytochemical staining containing 10% animal serum to a concentration of 0.1 to 50 µg/ml.

[0200] The diluted antibody is put into the wells of the above 96-well plate in an amount of 20 to 500 µl/well, and the plate is allowed to stand under ice cooling for 30 minutes.

[0201] When the unlabeled monoclonal antibody is used, a buffer for immunocytochemical staining is added to the above plate to wash the cells. To the wells of the plate is added a buffer for immunocytochemical staining containing 0.1 to 50 µg/ml anti-mouse immunoglobulin antibody or anti-rat immunoglobulin antibody labeled with a fluorescent dye such as FITC (fluorescein isothiocyanate) or phycoerythrin in an amount of 50 to 500 µl/well. Then, the plate is allowed to stand in the dark under ice cooling for 30 minutes.

[0202] When the biotin-labeled monoclonal antibody is used, streptoavidin labeled with a fluorescent dye such as FITC or phycoerythrin is added to the wells of the above plate in an amount of 50 to 500 μ l/well. Then, the plate is allowed to stand in the dark under ice cooling for 30 minutes.

[0203] In both cases, after the plate is allowed to stand, a buffer for immunocytochemical staining is added to the plate and the cells are washed well, followed by analysis using a fluorescence microscope, a cell sorter, or the like.

[0204] The expression-regulating compound can be identified by searching for a test sample increasing or decreasing the content of the polypeptide of the present invention as compared with the system without the addition of the test sample.

[0205] A substance increasing the content of the polypeptide of the present invention can be used similarly to the agonist. A substance decreasing the content of the polypeptide of the present invention can be used as the antagonist.

(2) Search and Identification Using a System for Determination of a Transcription Product of the DNA Encoding the Polypeptide of the Present Invention

[0206] The expression-regulating compound can be searched for and identified by contacting a test sample with cells expressing the polypeptide of the present invention or the mRNA encoding the polypeptide and then determining the content of the mRNA.

[0207] As the cells expressing the polypeptide of the present invention or the mRNA encoding the polypeptide, the cell lines described in [6](1) above, etc. can be used. As the test sample, the test samples mentioned in [5] above can be used.

[0208] The cells expressing the polypeptide of the present invention or the mRNA encoding the polypeptide are suspended in a medium allowing the growth of the cells, and a test sample is added to the medium for the contact with the cells. Then, the content of the mRNA expressed in the cells is determined by ordinary Northern hybridization, RNA dot blotting hybridization, RT-PCR, or the like.

[0209] Probes useful in the hybridization and primers useful in the RT-PCR include DNA fragments encoding the polypeptide of the present invention.

[0210] Specifically, an oligonucleotide having a nucleotide sequence identical with a sequence of 5 to 60 consecutive nucleotides in the nucleotide sequence selected from the group consisting of the nucleotide sequences shown in SEQ ID NOS: 2, 23, 27 and 39, and an oligonucleotide having a nucleotide sequence complementary to that of said oligonucleotide can be preferably used.

[0211] The expression-regulating compound can be identified by searching for a test sample increasing or decreasing the content of the mRNA encoding the polypeptide of the present invention as compared with the system without the addition of the test sample.

[0212] A substance increasing the content of the mRNA encoding the polypeptide of the present invention can be used as the agonist. A substance decreasing the content of the mRNA encoding the polypeptide of the present invention can be used as the antagonist.

(3) Search and Identification Using a Reporter Gene

[0213] The expression-regulating compound can be searched for and identified by contacting a test sample with a transformant transformed with a plasmid containing DNA in which a reporter gene is ligated downstream of the region regulating the transcription of the DNA encoding the polypeptide of the present invention (hereinafter referred to as the transcription-regulating region), and then determining the amount of the expressed polypeptide encoded by the reporter gene.

[0214] The transcription-regulating region is usually present in the 5' upstream region of DNA. The 5' upstream region of the DNA encoding the polypeptide of the present invention can be prepared, for example, by using Genome Walker Kits (Clontech). The region may be cleaved with appropriate restriction enzymes to obtain a fragment of appropriate length, which can also be used as the transcription-regulating region.

[0215] The reporter gene may be any DNA so far as its translation product is stable in cells and the amount of the translation product can be easily determined. Examples of the polypeptides encoded by such DNAs include chloramphenicol acetyltransferase (CAT), β -galactosidase (β -gal), luciferase (luc), β -glucuronidase, aequorin and green fluorescent protein (GFP).

[0216] Any cell can be used as the host cell for introducing the reporter plasmid containing the transcription-regulating region. Preferred are the cell lines confirmed to express the polypeptide of the present invention or the mRNA encoding the polypeptide described in [6](1) above.

[0217] As the test sample, the test samples mentioned in [5] above can be used.

[0218] The reporter gene is ligated downstream of the transcription-regulating region by a conventional method, and the obtained plasmid is used for transformation of host cells according to a conventional method.

[0219] It is also possible to prepare a gene targeting vector by ligating a positive selection marker (e.g., G418 resistance gene) or a negative selection marker (e.g., herpes simplex virus thymidine kinase gene and diphtheria toxin A fragment gene) and thereby to prepare cell lines in which a part of the chromosomal DNA encoding the polypeptide of the present invention is replaced by the reporter gene [Nature, 336, 348 (1988); Analytical Biochemistry, 214, 77 (1993); Gene Targeting, The Practical Approach Series, IRL Press (1993)].

[0220] The obtained transformant is suspended in a medium allowing the growth of the transformant cells, and a test sample is added to the medium for the contact with the cells. Then, the amount of the polypeptide encoded by the reporter gene which was expressed in the cells is detected and determined by a method suitable for the polypeptide.

[0221] The detection and determination can be carried out, for example, by the method described in Molecular Cloning, Second Edition, Chapter 16, page 60 in the case of CAT, the method described in Molecular Cloning, Second Edition, Chapter 16, page 66 in the case of β -gal, the method described in Experimental Medicine, Supplement, Bio Manual Series 4, Methods for Gene Introduction, Expression and Analysis, 89 (1994) in the case of luc, and the method described in Proc. Natl. Acad. Sci. USA, 94, 4653 (1997) in the case of GFP.

[0222] The expression-regulating compound can be identified by searching for a test sample increasing or decreasing the content of the polypeptide encoded by the reporter gene as compared with the system without the addition of the test sample.

[0223] A substance increasing the content of the polypeptide encoded by the reporter gene can be used as the agonist. A substance decreasing the content of the polypeptide encoded by the reporter gene can be used as the antagonist.

[7] Utilization of the DNA, Polypeptide, Antibody, Agonist, Antagonist and Expression-regulating Compound of the Present Invention

[0224]

(1) The DNA of the present invention can be used as a probe in Northern hybridization on RNA extracted from tissue or cells of a human or a non-human mammal such as mouse in the same manner as in [1] (1) above to detect or determine the mRNA encoding the polypeptide of the present invention in the tissue or cells.

By comparing the expression levels of the mRNA in various tissues, the topographical pattern of expression of the polypeptide of the present invention can be clarified.

(2) The oligonucleotide of the present invention can be used as a specific primer for the DNA of the present invention in RT-PCR [reverse transcription PCR; PCR Protocols (1990)] on RNA extracted from tissue or cells of a human or a non-human mammal such as mouse in the same manner as in 1 above to detect or determine the mRNA encoding the polypeptide of the present invention.

The method for determining the RNA can be applied to the diagnosis of a disease in which the DNA of the present invention is concerned.

By determining the mRNA in animal models of various diseases, the importance of the DNA product in the diseases can be clarified. Further, evaluation of a drug can be made based on the comparison of expression levels of the mRNA in the presence and absence of the drug.

(3) The oligonucleotide of the present invention can be used as a probe in *in situ* hybridization [Methods in Enzymology, 254, 419 (1995)] on a tissue section taken from a human or a non-human mammal such as mouse to obtain more detailed information on the expression pattern, for example, to specify the cells expressing the polypeptide of the present invention in the tissue.

The information thus obtained as to which tissue or cells express the polypeptide of the present invention and what stimulation to the cells causes a change in expression level is useful for analyzing the physiological functions of the polypeptide of the present invention and its participation in diseases.

(4) The DNA of the present invention can be used as a probe in Southern hybridization (Molecular Cloning, Second Edition) on genomic DNA to detect a mutation in the DNA encoding the polypeptide of the present invention.

The detection of the mutation enables diagnosis of diseases considered to be causable by the mutation in the DNA, for example, asthma, ischemic diseases, arthritis, rheumatism, sepsis, dermatitis, arteriosclerosis, pain, Parkinson disease, Alzheimer disease, malignant tumor, nephritis, diabetes and ischemic reperfusion injury.

(5) By analysis of the nucleotide sequence of the DNA encoding the polypeptide of the present invention after amplification by PCR or by analysis using a DNA chip, polymorphisms such as single nucleotide polymorphisms (SNP) can be detected. The detection of the polymorphisms enables diagnosis of diseases considered to be as-

sociated with the polymorphisms of the DNA, for example, asthma, ischemic diseases, arthritis, rheumatism, sepsis, dermatitis, arteriosclerosis, pain, Parkinson disease, Alzheimer disease, malignant tumor, nephritis, diabetes and ischemic reperfusion injury.

(6) The antisense oligonucleotide (RNA, DNA or a derivative thereof) of the present invention can be used for repressing the transcription of the DNA encoding the polypeptide of the present invention or the translation of the mRNA [Chemistry, 46, 681 (1991); Bio/Technology, 9, 358 (1992)] and thereby for preventing or treating diseases the occurrence of which is considered to be associated with the DNA, for example, asthma, ischemic diseases, arthritis, rheumatism, sepsis, dermatitis, arteriosclerosis, pain, Parkinson disease, Alzheimer disease, malignant tumor, nephritis, diabetes and ischemic reperfusion injury.

The above antisense oligonucleotide is designed and prepared on the basis of an oligonucleotide having a nucleotide sequence complementary to a sequence of 5 to 60 consecutive nucleotides in the nucleotide sequence of the DNA encoding the polypeptide of the present invention, preferably, an oligonucleotide having a nucleotide sequence complementary to a sequence of 5 to 60 consecutive nucleotides in the translation initiation region of the DNA encoding the polypeptide of the present invention, and is administered to a living organism.

The pharmaceutical comprising the DNA of the present invention can be prepared in the same manner as in the preparation of pharmaceutical preparations comprising the agonist or antagonist of the polypeptide of the present invention described in [5] above. The obtained pharmaceutical preparation can be administered in the same manner as in [5] above.

(7) The polypeptide of the present invention can be obtained according to the method described in [2] above using the DNA of the present invention.

The polypeptide of the present invention can be used as a therapeutic agent or a preventing agent for diseases such as asthma, ischemic diseases, arthritis, rheumatism, sepsis, dermatitis, arteriosclerosis, pain, Parkinson disease, Alzheimer disease, malignant tumor, nephritis, diabetes and ischemic reperfusion injury.

The pharmaceutical comprising the polypeptide of the present invention can be prepared in the same manner as in the preparation of pharmaceutical preparations comprising the agonist or antagonist of the polypeptide of the present invention described in [5] above. The obtained pharmaceutical preparation can be administered in the same manner as in [5] above.

(8) The oligonucleotide of the present invention, either single-stranded or double-stranded, can be inserted into a virus vector (e.g., retrovirus, adenovirus and adeno-associated virus) and other vectors to prepare vectors for gene therapy.

(9) The antibody to the polypeptide of the present invention can be produced according to the method described in [3] above using the polypeptide of the present invention as an antigen.

The antibody to the polypeptide of the present invention can be used for immunological detection or determination of the polypeptide of the present invention.

The detection or determination can be carried out by methods such as ELISA using a microtiter plate, immunohistochemical staining by the enzyme-labeled antibody technique or the fluorescent antibody technique, and the detection method using Western blotting.

Specifically, useful methods include sandwich ELISA using two kinds of monoclonal antibodies recognizing different epitopes wherein the antibodies are selected from the antibodies reacting with the polypeptide of the present invention in a liquid phase, and radioimmunoassay using the polypeptide of the present invention labeled with a radioisotope such as ^{125}I and an antibody recognizing the polypeptide of the present invention.

The antibody of the present invention can also be used for immunohistochemical staining using histologic sections.

The polypeptide of the present invention existing in cells or tissues of healthy individuals and subjects can be immunologically detected or determined using the antibody of the present invention. Comparison of the expression level of the polypeptide between the healthy individuals and subjects is useful for the pathologic diagnosis of diseases such as asthma, ischemic diseases, arthritis, rheumatism, sepsis, dermatitis, arteriosclerosis, pain, Parkinson disease, Alzheimer disease, malignant tumor, nephritis, diabetes and ischemic reperfusion injury, of the subjects.

Further, the polypeptide existing in cells or tissues of animal models of various diseases can be immunologically detected or determined using the antibody of the present invention. By comparing the result with that on normal animals, the importance of the polypeptide in the diseases can be clarified. Furthermore, evaluation of a drug can be made based on the comparison of expression levels of the polypeptide in the presence and absence of the drug.

(10) Administration of the antibody inhibiting the function of the polypeptide of the present invention (phospholipase A₂ activity) is effective for the treatment or prevention of diseases such as asthma, ischemic diseases, arthritis, rheumatism, sepsis, dermatitis, arteriosclerosis, pain, Parkinson disease, Alzheimer disease, malignant tumor, nephritis, diabetes and ischemic reperfusion injury.

5 The pharmaceutical comprising the antibody of the present invention can be prepared in the same manner as in the preparation of pharmaceutical preparations comprising the agonist or antagonist of the polypeptide of the present invention described in [5] above. The obtained pharmaceutical preparation can be administered in the same manner as in [5] above.

10 Certain embodiments of the invention are illustrated in the following examples, which are not to be construed as limiting the scope of the invention.

In the following examples, phospholipase A₂ and cytoplasmic phospholipase A₂ are abbreviated as PLA₂ and cPLA₂, respectively.

Brief Description of the Drawings

[0225]

Fig. 1 shows construction of plasmid p5269+C5.

20 Fig. 2 shows comparison between the amino acid sequence of the human-derived polypeptide of the present invention having the amino acid sequence shown in SEQ ID NO: 1 (upper lines: positions 121 to 476) and that of human cPLA₂α (GenBank: AAA60105) (lower lines: positions 1 to 309). Asterisks indicate identical amino acid residues and periods indicate similar amino acid residues. (Amino acid residues are shown by one letter notation.) GXSGS motif is indicated by an underline.

25 Fig. 3 is a continuation of Fig. 2 and shows comparison between the amino acid sequence of the human-derived polypeptide of the present invention having the amino acid sequence shown in SEQ ID NO: 1 (upper lines: positions 477 to 849) and that of human cPLA₂α (GenBank: AAA60105) (lower lines: positions 310 to 729). Asterisks indicate identical amino acid residues and periods indicate similar amino acid residues. (Amino acid residues are shown by one letter notation.)

30 Fig. 4 shows comparison between the amino acid sequence of the human-derived polypeptide of the present invention having the amino acid sequence shown in SEQ ID NO: 1 (upper lines: positions 1 to 400) and that of human cPLA₂β (GenBank: AAC78836) (lower lines: positions 181 to 571). Asterisks indicate identical amino acid residues and periods indicate similar amino acid residues. (Amino acid residues are shown by one letter notation.) GXSGS motif is indicated by an underline.

35 Fig. 5 is a continuation of Fig. 4 and shows comparison between the amino acid sequence of the human-derived polypeptide of the present invention having the amino acid sequence shown in SEQ ID NO: 1 (upper lines: positions 401 to 849) and that of human cPLA₂β (GenBank: AAC78836) (lower lines: positions 572 to 1012). Asterisks indicate identical amino acid residues and periods indicate similar amino acid residues. (Amino acid residues are shown by one letter notation.)

40 Fig. 6 shows the results when PCR primers were designed based on the information on the nucleotide sequence of cDNA encoding the human-derived polypeptide of the present invention, and PCR was carried out using cDNAs prepared from mRNAs of various human organs as templates. The results obtained by subjecting amplified products to agarose gel electrophoresis are shown. "-" represents control (no cDNA addition).

Fig. 7 shows the steps for constructing plasmid p600-N and its restriction map.

45 Fig. 8 shows the results of Northern hybridization carried out on a poly(A)⁺ RNA filter [filter for Human Multiple Tissue Northern Blots (Clontech)] of human heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas using a partial nucleotide sequence (about 0.6 kb) of cDNA encoding the human-derived polypeptide of the present invention as a probe.

Fig. 9 shows the steps for constructing plasmid pPLAH-1393 and its restriction map.

50 Fig. 10 shows the results of measurement of the PLA₂ activity in the soluble fraction of insect cells expressing the human-derived polypeptide of the present invention. "1393" represents insect cells infected with a virus prepared only from a vector, and PLAH represents insect cells expressing the human-derived polypeptide of the present invention. The numbers on the abscissa indicate the amount of the polypeptide (μg), and those on the ordinate indicate PLA₂ activity (dmp).

55 Fig. 11 shows comparison between the amino acid sequence of the human-derived polypeptide of the present invention (upper lines: positions 1 to 300) and that of the mouse-derived polypeptide of the present invention (lower lines: positions 1 to 296). Asterisks indicate identical amino acid residues and periods indicate similar amino acid residues. (Amino acid residues are shown by one letter notation.)

Fig. 12 shows comparison between the amino acid sequence of the human-derived polypeptide of the present

invention (upper lines: positions 301 to 539) and that of the mouse-derived polypeptide of the present invention (lower lines: positions 297 to 536). Asterisks indicate identical amino acid residues and periods indicate similar amino acid residues. (Amino acid residues are shown by one letter notation.)

Fig. 13 shows comparison between the amino acid sequence of the human-derived polypeptide of the present invention (upper lines: positions 540 to 849) and that of the mouse-derived polypeptide of the present invention (lower lines: positions 537 to 854). Asterisks indicate identical amino acid residues and periods indicate similar amino acid residues. (Amino acid residues are shown by one letter notation.)

Fig. 14 shows comparison between the amino acid sequence of the human-derived polypeptide of the present invention (lower lines) and the partial amino acid sequence of the rat-derived polypeptide of the present invention (upper lines). Asterisks indicate identical amino acid residues and periods indicate similar amino acid residues.

Fig. 15 shows the results when PCR primers were designed based on the information on the nucleotide sequence of cDNA encoding the mouse- or rat-derived polypeptide of the present invention; PCR was carried out using cDNAs prepared from mRNAs of various organs of mouse or rat as templates; and the amplified products were subjected to agarose gel electrophoresis. "-" represents control (no cDNA addition).

Fig. 16 shows comparison among the amino acid sequence of the human-derived polypeptide of the present invention (upper lines: positions 1 to 473), that of the mouse-derived polypeptide of the present invention (middle lines: positions 1 to 470) and that of the BALB/C mouse-derived polypeptide of the present invention (lower lines: positions 1 to 469). Asterisks indicate identical amino acid residues and periods indicate similar amino acid residues. (Amino acid residues are shown by one letter notation.)

Fig. 17 is a continuation of Fig. 16 and shows comparison among the amino acid sequence of the human-derived polypeptide of the present invention (upper lines: positions 474 to 849), that of the mouse-derived polypeptide of the present invention (middle lines: positions 471 to 854) and that of the BALB/C mouse-derived polypeptide of the present invention (lower lines: positions 470 to 853). Asterisks indicate identical amino acid residues and periods indicate similar amino acid residues. (Amino acid residues are shown by one letter notation.)

Fig. 18 shows the steps for constructing plasmid pmPLAH-1393 and its restriction map.

Fig. 19 shows the results of measurement of the PLA₂ activity in the soluble fraction of insect cells infected with a virus. m-1393V represents insect cells infected with a virus prepared only from a vector, and m-cPLA2H represents insect cells expressing the mouse-derived polypeptide of the present invention.

Fig. 20 shows the results of measurement of the calcium concentration dependency of PLA₂ activity in the soluble fraction of insect cells infected with a virus. m-1393V represents insect cells infected with a virus prepared only from a vector, and m-cPLA2H represents insect cells expressing the mouse-derived polypeptide of the present invention.

Fig. 21 shows the results of measurement of the reaction time dependency of PLA₂ activity in the soluble fraction of insect cells infected with a virus. m-1393V represents insect cells infected with a virus prepared only from a vector, and m-cPLA2H represents insect cells expressing the mouse-derived polypeptide of the present invention.

Fig. 22 shows the results when PCR primers were designed based on the information on the nucleotide sequences of DNA encoding the human-derived polypeptide of the present invention, human cPLA₂α and human G3PDH; PCR was carried out using cDNAs prepared from RNAs of cultured human cell lines (K-562, HL-60, Jurkat, 293EBNA, DU145, PC-3 and LNCaP.FGS) as templates; and the amplified products were subjected to agarose gel electrophoresis.

Fig. 23 shows the results of Northern hybridization carried out on a poly(A)⁺RNA filter [Human Fetal Normal Tissue mRNA Northern Blot II (Biochain)] of human fetal heart, kidney, skin and small intestine and adult lung using a partial nucleotide sequence (about 0.6 kb) of cDNA encoding the human-derived polypeptide of the present invention as a probe.

Explanation of Symbols

[0226]

kb: Kilobase pairs
Ap: Ampicillin resistance gene
T7: T7 promoter
BAP: Bacterial alkaline phosphatase
Flag: Flag tag

Best Modes for Carrying Out the InventionExample 1

5 Cloning of cDNA Encoding the Human-derived Polypeptide of the Present Invention

[0227] Unless otherwise noted, the genetic engineering techniques in the following examples were carried out according to the known methods described in Molecular Cloning, Second Edition.

10 (1) Preparation of a cDNA Library Derived from Human Small Intestine

[0228] Total RNA was extracted from human small intestine using an RNA extraction kit (#27-9270-01) produced by Pharmacia. Thereafter, mRNA was extracted and purified in accordance with the polyA(+)RNA purification method described in literature [J. Sambrook, E.F. Fritsch & T. Maniatis, Molecular Cloning Second Edition, Cold Spring Harbor Laboratory Press (1989)].

[0229] A cDNA library was prepared from each of polyA(+)RNA according to the oligo-cap method [Gene, 138, 171 (1994)]. BAP (bacterial alkaline phosphatase) treatment, TAP (tobacco acid pyrophosphatase) treatment, RNA ligation, single-stranded cDNA synthesis and RNA removal were carried out according to the literature [Tanpakushitsu, Kaku-san, Koso (Protein, Nucleic Acid, and Enzyme), 41, 197 (1996); Gene, 200, 149 (1997)] using an oligo-cap linker (SEQ ID NO: 5) and an oligo dT primer (SEQ ID NO: 6).

[0230] After conversion to double-stranded cDNA by PCR (polymerase chain reaction) using primers corresponding to the 5'- and 3'-ends (SEQ ID NOS: 7 and 8), the cDNA was cleaved with restriction enzyme SfiI. The resulting cDNA was incorporated into a vector, pME18SFL3 (GenBank AB009864, Expression vector, 3392 bp) previously cleaved with DraIII to prepare a cDNA library. The cDNA was incorporated in one direction to enable expression.

25 (2) Random Sequencing

[0231] Plasmid DNA was obtained from each Escherichia coli clone in the cDNA library prepared in (1) above according to a conventional method, and the nucleotide sequence at the 5'-end of the cDNA contained in each plasmid was determined. Determination of the nucleotide sequence was carried out using a kit (BigDye Terminator Cycle Sequencing FS Ready Reaction Kit, PE Biosystems) and a DNA sequencer (ABI PRISM 377, PE Biosystems). DNAs having the nucleotide sequences shown in SEQ ID NOS: 9 and 10, respectively, were synthesized and used as primers.

35 (3) Analysis Using Homology Search Software

[0232] The nucleotide sequences obtained were analyzed using BLAST SEARCH homology search software to find a nucleotide sequence which was recognized to be homologous to cPLA₂. Determination of the entire nucleotide sequence of the clone (c-hsi05269) which was considered to have the above nucleotide sequence revealed that plasmid c-hsi05269 contained cDNA having the nucleotide sequence of about 1.5 kb shown in SEQ ID NO: 4. The amino acid sequence of the novel polypeptide encoded by the nucleotide sequence is shown in SEQ ID NO: 3.

40 (4) Cloning of cDNA Entirely Containing the Region Homologous to cPLA₂

[0233] DNA primers having the nucleotide sequences shown in SEQ ID NOS: 11 and 12, respectively, were designed based on the information on the nucleotide sequence obtained in (3) above, and the C-terminal region was amplified by PCR using Human Kidney Marathon-Ready cDNA kit (Clontech) according to the following method.

[0234] That is, PCR was carried out using 20 µl of a reaction solution containing 2 µl of Human Kidney Marathon-Ready cDNA, 0.2 µmol/l each of the DNA primer having the nucleotide sequence shown in SEQ ID NO: 11 and AP1 primer (attached to the kit), a mixed solution of dNTPs (dATP, dGTP, dCTP and dTTP) containing 200 µmol/l each of the components, 0.5 µl of a mixed solution of Advantage 2 polymerase (Clontech) and 1 x Advantage 2 PCR buffer under the following conditions.

[0235] That is, using a thermal cycler, PTC-200 (MJ Research), PCR was carried out, after heating at 95°C for 3 minutes, by 5 cycles, one cycle consisting of reaction at 94°C for 15 seconds and reaction at 72°C for 4 minutes; by 5 cycles, one cycle consisting of reaction at 94°C for 15 seconds and reaction at 70°C for 4 minutes; and by 20 cycles, one cycle consisting of reaction at 94°C for 15 seconds and reaction at 68°C for 4 minutes. Subsequently, PCR was carried out using 50 µl of a reaction solution containing 5 µl of 100-fold dilution of the obtained PCR reaction mixture, 0.2 µmol/l each of the DNA primer having the nucleotide sequence shown in SEQ ID NO: 12 and AP2 primer (attached to the kit), a mixed solution of dNTPs (dATP, dGTP, dCTP and dTTP) containing 200 µmol/l each of the components,

1 µl of a mixed solution of Advantage 2 polymerase and 1 x Advantage 2 PCR buffer under the following conditions.

[0236] That is, using a thermal cycler, PTC-200, PCR was carried out, after heating at 95°C for 3 minutes, by 30 cycles, one cycle consisting of reaction at 94°C for 15 seconds and reaction at 68°C for 4 minutes. A 5 µl aliquot of the resulting PCR reaction mixture was subjected to agarose gel electrophoresis to confirm that an about 2.5 kb DNA fragment was amplified. The DNA fragment was then purified using QIAEX II Gel Extraction Kit (QIAGEN) according to the manual.

[0237] The obtained DNA fragment (50 ng) and 50 ng of pCR2.1 T-Vector (Invitrogen) were subjected to ligation using DNA Ligation Kit Ver. 2 (Takara Shuzo) according to the manual to obtain a recombinant plasmid DNA. *Escherichia coli* JM109 was transformed using the recombinant plasmid DNA, and plasmid pPL-C was obtained according to a conventional method.

[0238] The nucleotide sequence of the DNA fragment contained in plasmid pPL-C was determined according to a conventional method, whereby it was found that the inserted DNA fragment was capable of ligation with the AccI site of c-hsi05269 at the AccI site of the inserted fragment.

[0239] Plasmid c-hsi05269 (2 µg) was dissolved in 50 µl of a buffer consisting of 10 mmol/l Tris-HCl (pH 7.5), 10 mmol/l magnesium chloride, 1 mmol/l dithiothreitol (hereinafter abbreviated to DTT) and 50 mmol/l sodium chloride, and digestion reaction was carried out at 37°C for 3 hours following the addition of 10 units of AccI (Takara Shuzo). After extraction with phenol and precipitation with ethanol, the precipitate obtained was dissolved in 50 µl of a buffer consisting of 50 mmol/l Tris-HCl (pH 7.5), 10 mmol/l magnesium chloride, 1 mmol/l DTT and 100 mmol/l sodium chloride, and digestion reaction was carried out at 37°C for 3 hours following the addition of 10 units of EcoRI (Takara Shuzo). The resulting reaction mixture was subjected to agarose gel electrophoresis, and the EcoRI-AccI fragment (1.3 kb) was purified using QIAEX II Gel Extraction Kit (QIAGEN).

[0240] Separately, 2 µg of plasmid pPL-C was dissolved in 50 µl of a buffer consisting of 10 mmol/l Tris-HCl (pH 7.5), 10 mmol/l magnesium chloride, 1 mmol/l DTT and 50 mmol/l sodium chloride, and digestion reaction was carried out at 37°C for 3 hours following the addition of 10 units of AccI (Takara Shuzo).

[0241] After extraction with phenol and precipitation with ethanol, the obtained precipitate was dissolved in 50 µl of a buffer consisting of 50 mmol/l Tris-HCl (pH 7.5), 10 mmol/l magnesium chloride, 1 mmol/l DTT and 100 mmol/l sodium chloride, and digestion reaction was carried out at 37°C for 3 hours following the addition of 10 units of NotI (Takara Shuzo). The resulting reaction mixture was subjected to agarose gel electrophoresis, and the AccI-NotI fragment (2.2 kb) was purified using QIAEX II Gel Extraction Kit (QIAGEN).

[0242] On the other hand, 2 µg of plasmid pBluescriptII KS(-) (STRATAGENE) was dissolved in 50 µl of a buffer consisting of 50 mmol/l Tris-HCl (pH 7.5), 10 mmol/l magnesium chloride, 1 mmol/l DTT and 100 mmol/l sodium chloride, and digestion reaction was carried out at 37°C for 6 hours following the addition of 10 units of EcoRI and NotI (Takara Shuzo). The resulting reaction mixture was subjected to agarose gel electrophoresis, and the EcoRI-NotI fragment (3.0 kb) was purified using QIAEX II Gel Extraction Kit (QIAGEN).

[0243] The EcoRI-AccI fragment (1.3 kb) (50 ng) derived from plasmid c-hsi05269, 50 ng of the AccI-NotI fragment (2.2 kb) derived from plasmid pPL-C and 50 ng of the EcoRI-NotI fragment (3.0 kb) derived from pBluescriptII KS(-) respectively obtained above were subjected to ligation using DNA Ligation Kit Ver. 2 (Takara Shuzo) according to the manual to obtain a recombinant plasmid DNA. *Escherichia coli* JM109 was transformed using the recombinant plasmid DNA, and plasmid p5269+C5 was obtained according to a conventional method. The steps for constructing the plasmid and its restriction map are shown in Fig. 1.

[0244] *Escherichia coli* JM109 carrying plasmid p5269+C5 was deposited under the Budapest Treaty with International Patent Organism Depository, National Institute of Advanced Industrial Science and Technology, Central 6, 1-1, Higashi 1-chome, Tsukuba-shi, Ibaraki, Japan (former name: National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki, Japan) on August 25, 2000 as *Escherichia coli* JM109/p5269+C5 (FERM BP-7281).

[0245] The nucleotide sequence resulting from the ligation had the nucleotide sequence shown in SEQ ID NO: 2 and encoded a novel polypeptide having the amino acid sequence shown in SEQ ID NO: 1.

[0246] As a result of Smith & Waterman search of known protein sequence databases (GenBank, etc.) for the amino acid sequence, homology to polypeptides of cPLA₂ family was strongly detected. Thus, the amino acid sequence was aligned with the amino acid sequence of human cPLA₂α (GenBank: AAA60105) and that of human cPLA₂β (GenBank: AAC78836).

[0247] Figs. 2 and 3 show the results of alignment with the human cPLA₂α sequence, and Figs. 4 and 5 show those with the human cPLA₂β sequence. GXSGS sequence (SEQ ID NO: 15), an amino acid sequence common to cPLA₂, was also observed (the underlined parts in Figs. 2 and 4).

Example 2

Analysis of Expression Using RT-PCR Method

[0248] A 5'-end DNA primer having the nucleotide sequence shown in SEQ ID NO: 13 and a 3'-end DNA primer having the nucleotide sequence shown in SEQ ID NO: 14 were designed and synthesized based on the information on the nucleotide sequence determined in Example 1.

[0249] PCR was carried out using 20 µl of a reaction solution containing 1.0 µmol/l each of the two primers (SEQ ID NOS: 13 and 14), 2 µl of a cDNA library prepared from each of the mRNAs of various human organs, a mixed solution of dNTPs (dATP, dGTP, dCTP and dTTP) containing 200 µmol/l each of the components, 2.5 units of Taq Gold polymerase (Perkin Elmer) and 1 x Taq Gold (Mg plus) buffer (Perkin Elmer) under the following conditions.

[0250] That is, using a thermal cycler, PTC-200 (MJ Research), PCR was carried out, after heating at 95°C for 10 minutes, by 35 cycles, one cycle consisting of reaction at 94°C for one minute and reaction at 60°C for one minute, followed by heating at 72°C for 8 minutes.

[0251] A 7 µl aliquot of the resulting PCR reaction mixture was subjected to agarose gel electrophoresis to confirm that an anticipated about 0.6 kb DNA fragment was amplified. Strong expression was observed in kidney, lung, prostate, thymus, thyroid, trachea and uterus. The results of electrophoresis are shown in Fig. 6.

Example 3

Analysis of Expression of mRNA by Northern Hybridization

[0252] PCR was carried out using 50 µl of a reaction solution containing 0.2 µmol/l each of the two primers (SEQ ID NOS: 13 and 14), a mixed solution of dNTPs (dATP, dGTP, dCTP and dTTP) containing 200 µmol/l each of the components, 2 µl of Human Kidney Marathon-Ready cDNA, 2.5 units of Ampli Taq Gold polymerase (Perkin Elmer) and 1 x Taq Gold buffer under the following conditions.

[0253] That is, using a thermal cycler, PTC-200, PCR was carried out, after heating at 95°C for 10 minutes, by 35 cycles, one cycle consisting of reaction at 94°C for one minute and reaction at 60°C for one minute, followed by heating at 72°C for 8 minutes.

[0254] A 5 µl aliquot of the resulting PCR reaction mixture was subjected to agarose gel electrophoresis to confirm that an about 0.6 kb DNA fragment was amplified. The DNA fragment was then purified using QIAEX II Gel Extraction Kit (QIAGEN) according to the attached manual.

[0255] The obtained DNA fragment (50 ng) and 50 ng of pT7Blue T-Vector were subjected to ligation using DNA Ligation Kit (Takara Shuzo) according to the manual to obtain a recombinant plasmid DNA. *Escherichia coli* JM109 was transformed using the recombinant plasmid DNA, and plasmid p600-N to be used for the preparation of a probe for Northern analysis was prepared according to a conventional method. The steps for constructing the plasmid and its restriction map are shown in Fig. 7.

[0256] Plasmid p400-N prepared (10 µg) was dissolved in 50 µl of a buffer consisting of 10 mmol/l Tris-HCl (pH 7.5), 10 mmol/l magnesium chloride, 50 mmol/l sodium chloride and 1 mmol/l DTT, and digestion reaction was carried out at 37°C for 6 hours following the addition of 30 units of *Bam*HI (Takara Shuzo). The reaction mixture was subjected to extraction with phenol-chloroform and precipitation with ethanol to recover a DNA fragment.

[0257] The DNA fragment (1 µg) was dissolved in 50 µl of a buffer containing 40 mmol/l Tris-HCl (pH 8.0), 6 mmol/l magnesium chloride, 2 mmol/l spermidine, 10 mmol/l DTT, 1 mmol/l ATP, 1 mmol/l CTP, 1 mmol/l GTP, 0.65 mmol/l UTP and 0.35 mmol/l digoxigenin-11-UTP, and *in vitro* transcription reaction was carried out at 37°C for 2 hours following the addition of 40 units of T7 RNA polymerase (Boehringer Mannheim).

[0258] After the reaction, a digoxigenin-labeled cRNA probe was recovered from the reaction mixture by precipitation with ethanol.

[0259] Using the probe, Northern hybridization was carried out on a poly(A)⁺RNA filter [filter for Human Multiple Tissue Northern Blots (Clontech)] of human heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas according to the following method.

[0260] The poly(A)⁺RNA filter of each of the organs was immersed in a buffer containing 50% formamide, 5-fold concentrated SSC (1-fold SSC consists of 150 mmol/l sodium chloride and 15 mmol/l sodium citrate), 0.5% sodium dodecyl sulfate (hereinafter abbreviated as SDS), 2% blocking reagent (Boehringer Mannheim) and 0.1 mg/ml salmon sperm DNA (hereinafter referred to as hybridization buffer), and prehybridization was performed at 70°C for 2 hours.

[0261] The filter was immersed in the hybridization buffer in which the above-mentioned digoxigenin-labeled cRNA probe was dissolved at a concentration of 1 g/ml, and hybridization was performed at 70°C for 15 hours.

[0262] The filter was washed once under the conditions of immersion in a buffer consisting of 2-fold concentrated SSC and 0.1% SDS at 70°C for 10 minutes and 3 times under the conditions of immersion in a buffer consisting of

0.2-fold concentrated SSC and 0.1% SDS at 70°C for 30 minutes.

[0263] The filter was further washed twice under the conditions of immersion in a buffer consisting of 100 mmol/l maleic acid (pH 7.5) and 150 mmol/l sodium chloride (hereinafter referred to as DIG I buffer) at room temperature for 15 minutes to remove SDS.

[0264] The resulting filter was immersed in a buffer consisting of 100 mmol/l maleic acid (pH 7.5), 150 mmol/l sodium chloride and 1% blocking reagent (hereinafter referred to as DIG II buffer), and blocking was performed at room temperature for one hour.

[0265] The filter was then immersed in a solution of alkaline phosphatase-labeled anti-digoxigenin antibody Fab fragment (Boehringer Mannheim) diluted 10000-fold with DIG II buffer and subjected to antigen-antibody reaction at room temperature for 30 minutes.

[0266] The resulting filter was washed three times under the conditions of immersion in DIG I buffer at room temperature for 30 minutes to remove excess antibody. Thereafter, the filter was immersed in a buffer consisting of 100 mmol/l Tris-HCl (pH 9.0), 100 mmol/l sodium chloride and 50 mmol/l magnesium chloride (hereinafter referred to as DIG III buffer) for 5 minutes to effect equilibration.

[0267] The filter was immersed in a solution of a light emitting substrate, CDP-Star (Boehringer Mannheim) diluted 100-fold with DIG III buffer at room temperature for 15 minutes to allow a signal to emit, and the signal was detected by a CCD camera (Fuji Photo Film).

[0268] The results are shown in Fig. 8. Bands of about 3.5 kb nucleotide and 6 kb nucleotide were observed in kidney and lung. Also, a band of about 3.5 kb nucleotide was observed in both skeletal muscle and heart.

Example 4

Expression of the Human-derived Polypeptide of the Present Invention Using an Insect Cell and Measurement of Phospholipase A₂ Activity of the Polypeptide

(1) Construction of Plasmid for the Preparation of Baculovirus

[0269] DNA primers having the nucleotide sequences shown in SEQ ID NOS: 16 and 17, respectively, were designed based on the nucleotide sequence obtained in Example 1 above and the N-terminal region into which Flag tag was inserted was amplified by PCR according to the following method.

[0270] PCR was carried out using 20 µl of a reaction solution containing 10 ng of plasmid c-hsi05269 obtained in Example 1 above, 0.3 µmol/l each of the primers having the nucleotide sequences shown in SEQ ID NOS: 16 and 17, respectively, a mixed solution of dNTPs (dATP, dGTP, dCTP and dTTP) containing 300 µmol/l each of the components, 1 mmol/l magnesium sulfate solution, 0.5 µl of Pfx DNA polymerase solution (Life Technologies) and 1 x Pfx DNA polymerase buffer under the following conditions.

[0271] That is, using a thermal cycler, PTC-200 (MJ Research), PCR was carried out, after heating at 95°C for 3 minutes, by 25 cycles, one cycle consisting of reaction at 94°C for one minute and reaction at 68°C for one minute, followed by reaction at 68°C for 5 minutes. A 5 µl aliquot of the resulting PCR reaction mixture was subjected to agarose gel electrophoresis to confirm that an about 1.4 kb DNA fragment was amplified. Thereafter, the DNA fragment was purified using QIAEX II Gel Extraction Kit (QIAGEN) according to the manual.

[0272] The obtained DNA fragment (50 ng) and 50 ng of T7Blue T-Vector (Novagen) were subjected to ligation using DNA Ligation Kit Ver. 2 (Takara Shuzo) according to the manual to obtain a recombinant plasmid DNA. *Escherichia coli* JM109 was transformed using the recombinant plasmid DNA, and plasmid pMF2 was obtained according to a conventional method.

[0273] On the other hand, DNA primers having the nucleotide sequence shown in SEQ ID NO: 18 and the nucleotide sequence shown in SEQ ID NO: 19 contained in plasmid pPL-C, respectively, were designed based on the nucleotide sequence obtained in Example 1 above, and the C-terminal region was amplified by PCR.

[0274] That is, PCR was carried out using 20 µl of a reaction solution containing 10 ng of plasmid pPL-C, 0.3 µmol/l each of the primers having the nucleotide sequences shown in SEQ ID NOS: 18 and 19, respectively, a mixed solution of dNTPs (dATP, dGTP, dCTP and dTTP) containing 300 µmol/l each of the components, 1 mmol/l magnesium sulfate solution, 0.5 µl of a mixed solution of Pfx DNA polymerase (Life Technologies) and 1 x Pfx DNA polymerase buffer under the following conditions.

[0275] That is, using a thermal cycler, PTC-200 (MJ Research), PCR was carried out, after heating at 95°C for 3 minutes, by 25 cycles, one cycle consisting of reaction at 94°C for one minute and reaction at 68°C for one minute, followed by reaction at 68°C for 5 minutes. A 5 µl aliquot of the PCR reaction mixture thus obtained was subjected to agarose gel electrophoresis to confirm that an about 1.5 kb DNA fragment was amplified. Thereafter, the DNA fragment was purified using QIAEX II Gel Extraction Kit (QIAGEN) according to the manual.

[0276] The obtained DNA fragment (50 ng) and 50 ng of T7Blue T-Vector (Novagen) were subjected to ligation using

DNA Ligation Kit Ver. 2 (Takara Shuzo) according to the manual to obtain a recombinant plasmid DNA. *Escherichia coli* JM109 was transformed using the recombinant plasmid DNA, and plasmid pC5PCR was obtained according to a conventional method.

[0277] The nucleotide sequences of DNA fragments contained in plasmid pMF2 and plasmid pC5PCR were determined according to a conventional method, and these inserted DNA fragments were subjected to ligation using the AccI site present in the inserted DNA fragments under the following conditions.

[0278] That is, 2 µg of plasmid pMF2 was dissolved in 50 µl of a buffer consisting of 20 mmol/l Tris-HCl (pH 8.5), 10 mmol/l magnesium chloride, 1 mmol/l DTT and 100 mmol/l potassium chloride, and digestion reaction was carried out at 37°C for 3 hours following the addition of 10 units of BamHI (Takara Shuzo). After extraction with phenol and precipitation with ethanol, the obtained precipitate was dissolved in 50 µl of a buffer consisting of 10 mmol/l Tris-HCl (pH 7.5), 10 mmol/l magnesium chloride, 1 mmol/l DTT and 50 mmol/l sodium chloride, and digestion reaction was carried out at 37°C for 3 hours following the addition of 10 units of AccI (Takara Shuzo). The resulting reaction mixture was subjected to agarose gel electrophoresis, and the BamHI-AccI fragment (1.3 kb) was purified using QIAEX II Gel Extraction Kit (QIAGEN).

[0279] Also, 2 µg of plasmid pCSPCR was dissolved in 50 µl of a buffer consisting of 50 mmol/l Tris-HCl (pH 7.5), 10 mmol/l magnesium chloride, 1 mmol/l DTT and 100 mmol/l sodium chloride, and digestion reaction was carried out at 37°C for 3 hours following the addition of 10 units of EcoRI (Takara Shuzo). After extraction with phenol and precipitation with ethanol, the obtained precipitate was dissolved in 50 µl of a buffer consisting of 10 mmol/l Tris-HCl (pH 7.5), 10 mmol/l magnesium chloride, 1 mmol/l DTT and 50 mmol/l sodium chloride, and digestion reaction was carried out at 37°C for 3 hours following the addition of 10 units of AccI (Takara Shuzo). The resulting reaction mixture was subjected to agarose gel electrophoresis, and the AccI-EcoRI fragment (1.4 kb) was purified using QIAEX II Gel Extraction Kit (QIAGEN).

[0280] On the other hand, 2 µg of plasmid pcDNA3.1 (Invitrogen) was dissolved in 50 µl of a buffer consisting of 20 mmol/l Tris-HCl (pH 8.5), 10 mmol/l magnesium chloride, 1 mmol/l DTT and 100 mmol/l potassium chloride, and digestion reaction was carried out at 37°C for 3 hours following the addition of 10 units of BamHI (Takara Shuzo). After extraction with phenol and precipitation with ethanol, the obtained precipitate was dissolved in 50 µl of a buffer consisting of 50 mmol/l Tris-HCl (pH 7.5), 10 mmol/l magnesium chloride, 1 mmol/l DTT and 100 mmol/l sodium chloride, and digestion reaction was carried out at 37°C for 3 hours following the addition of 10 units of EcoRI (Takara Shuzo). The resulting reaction mixture was subjected to agarose gel electrophoresis, and the BamHI-EcoRI fragment (5.4 kb) was purified using QIAEX II Gel Extraction Kit (QIAGEN).

[0281] The plasmid pMF2-derived BamHI-AccI fragment (1.3 kb) (50 ng), 50 ng of the plasmid pC5PCR-derived AccI-EcoRI fragment (1.4 kb) and 50 ng of the plasmid pcDNA3.1-derived BamHI-EcoRI fragment (5.4 kb) obtained above were subjected to ligation using DNA Ligation Kit Ver. 2 (Takara Shuzo) according to the manual to obtain a recombinant plasmid DNA. *Escherichia coli* JM109 was transformed using the recombinant plasmid DNA, and plasmid pPLAH-3.1 was obtained according to a conventional method.

[0282] Subsequently, 2 µg of plasmid pPLAH-3.1 was dissolved in 50 µl of a buffer consisting of 20 mmol/l Tris-HCl (pH 8.5), 10 mmol/l magnesium chloride, 1 mmol/l DTT and 100 mmol/l potassium chloride, and digestion reaction was carried out at 37°C for 3 hours following the addition of 10 units of BamHI (Takara Shuzo). The resulting reaction mixture was subjected to agarose gel electrophoresis, and the BamHI fragment (2.7 kb) was purified using QIAEX II Gel Extraction Kit (QIAGEN).

[0283] On the other hand, 2 µg of plasmid pVL1393 (PharMingen) was dissolved in 50 µl of a buffer consisting of 20 mmol/l Tris-HCl (pH 8.5), 10 mmol/l magnesium chloride, 1 mmol/l DTT and 100 mmol/l potassium chloride, and digestion reaction was carried out at 37°C for 3 hours following the addition of 10 units of BamHI (Takara Shuzo). After extraction with phenol and precipitation with ethanol, the obtained precipitate was dissolved in 30 µl of a buffer consisting of 50 mmol/l Tris-HCl (pH 9.0) and 1 mmol/l magnesium chloride, and dephosphorylation reaction was carried out at 60°C for 30 minutes following the addition of 0.5 unit of alkaline phosphatase (Takara Shuzo; derived from *E. coli* C75). The resulting reaction mixture was subjected to agarose gel electrophoresis, and a BamHI-alkaline phosphatase-treated fragment (9.6 kb) was purified using QIAEX II Gel Extraction Kit (QIAGEN). The plasmid pPLAH-3.1-derived BamHI fragment (2.7 kb) (50 ng) recovered above and 50 ng of the plasmid pVL1393-derived, BamHI-alkaline phosphatase-treated fragment (9.6 kb) were subjected to ligation using DNA Ligation Kit Ver. 2 (Takara Shuzo) according to the manual to obtain a recombinant plasmid DNA. *Escherichia coli* JM109 was transformed using the recombinant plasmid DNA, and plasmid pPLAH-1393 was obtained according to a conventional method. Fig. 9 shows the steps for constructing the plasmid and its restriction map.

(2) Preparation of Recombinant Baculovirus

[0284] Preparation of a virus was carried out according to the method described in the Baculovirus Expression Vector System Manual (PharMingen).

[0285] That is, 2×10^6 Sf9 cells were seeded on a petri dish of 6 cm in diameter and, after adhesion, the medium was replaced with a serum-free medium (Sf-900IISFM purchased from Life Tech). A mixed solution of DNA and lipofectin (24 μ l) containing 5 μ g of plasmid pPLAH-1393 prepared in (1) above or pVL1393, 15 ng of Linearized Baculogold DNA (PharMingen) and 6 ng of lipofectin solution (Life Technologies) was added to the above petri dish containing the serum-free medium so as to distribute evenly, and culturing was carried out at 27°C for 4 days. Following the addition of 2 ml of a medium containing serum (Esf921 purchased from Asahi Techno Glass), culturing was further carried out at 27°C for 3 days. After recovering the cells, the cell free culture was centrifuged at 800 rpm for 5 minutes to obtain a supernatant. The supernatant was added to the Sf9 cells adhered and culturing was carried out at 27°C for 3 days. After recovering the cells, the culture was centrifuged at 800 rpm for 5 minutes to obtain a supernatant containing a virus.

(3) Preparation of the Soluble Fraction of Insect Cells Expressing the Polypeptide

[0286] The supernatant containing the virus recovered in (2) above (2 ml) was added to 28 ml of 1.5×10^6 /ml suspending Sf9 cells, and the cells were cultured at 27°C for 4 days in the suspending state. The cells were recovered by centrifugation at 800 rpm for 5 minutes and washed with phosphate-buffered saline (PBS). The resulting cells were suspended in a buffer consisting of 25 mmol/l Tris-HCl (pH 7.5), 140 mmol/l sodium chloride, 5 mmol/l potassium chloride, 2 mmol/l EDTA and 1 x complete, EDTA-free (Boehringer Mannheim) and disrupted on ice using a sonicator. The extract was centrifuged at 15,000 rpm for 15 minutes, and the supernatant was used for the measurement of PLA₂ activity.

(4) Measurement of PLA₂ Activity

[0287] One hundred μ l of a reaction solution [100 mmol/l Tris-HCl (pH 7.5), 4 mmol/l calcium chloride, 1 mg/ml bovine serum albumin (substantially fatty acid-free, Sigma) and 8 μ mol/l Triton X-100] containing 1-palmitoyl-2-[1-¹⁴C]arachidonyl-phosphatidylcholine (48 mCi/mmol, Daiichi Kagaku Yakuhin) (2 μ mol/l) and the supernatant obtained above was incubated at 37°C for 2 hours, followed by the addition of Dole reagent (containing 2-propanol, heptane and sulfuric acid at a ratio of 78:20:2) to stop the reaction. To the reaction mixture were further added 0.3 ml of heptane and 0.2 ml of water, and mixing was effected by rotation. The resulting mixture was centrifuged at 3,000 rpm for 5 minutes, and 0.32 ml of the obtained upper layer was transferred to a tube containing 40 mg of silica gel (Silica gel 60, Merck), followed by addition of 0.3 ml of heptane. After mixing by rotating the tube, the mixture was centrifuged at 3,000 rpm for 5 minutes. A 400 μ l aliquot of the supernatant was transferred to a scintillation vial containing 3 ml of Ultima Gold (Packard), and the radioactivity was measured using a liquid scintillation counter (Beckman LS6500). The amount of the polypeptide was determined using the Bio Rad Protein Assay method. As a control, the soluble fraction of insect cells to which a virus prepared from plasmid pVL1393 had been introduced was used. The results are shown in Fig. 10.

[0288] The results shown in Fig. 10 demonstrated that the human-derived polypeptide of the present invention obtained in Example 1 above has PLA₂ activity that hydrolyzes the ester bond at the sn-2-position in 1-palmitoyl-2-arachidonyl-phosphatidylcholine.

Example 5

Cloning of DNA Encoding the Mouse-derived Polypeptide of the Present Invention

[0289] Based on the information on the nucleotide sequence of the DNA encoding the human-derived polypeptide of the present invention that was shown to have PLA₂ activity in Example 4 above, analysis was carried out using BLAST Search homology search software, and EST sequence (Genbank ACCESSION BF299949) to which homology was recognized was found. The clone was obtained (Cosmobio), and the entire nucleotide sequence was determined. As a result, it was found that plasmid pBF299949 contained cDNA having a nucleotide sequence highly homologous to the nucleotide sequence shown in SEQ ID NO: 4.

[0290] DNA primers having the nucleotide sequences shown in SEQ ID NOS: 20 and 21, respectively, were designed based on the information on the nucleotide sequence, and the N-terminal region was amplified by PCR using Mouse Lung Marathon-Ready cDNA kit (Clontech) according to the following method.

[0291] That is, PCR was carried out using 20 μ l of a reaction solution containing 2 μ l of Mouse Lung Marathon-Ready cDNA, 0.2 μ mol/l each of the primer having the nucleotide sequence shown in SEQ ID NO: 20 and AP1 primer (attached to the kit), a mixed solution of dNTPs (dATP, dGTP, dCTP and dTTP) containing 200 μ mol/l each of the components, 0.5 μ l of a mixed solution of Advantage 2 polymerase (Clontech) and 1 x Advantage 2 PCR buffer under the following conditions.

[0292] That is, using a thermal cycler, PTC-200 (MJ Research), PCR was carried out, after heating at 95°C for 3 minutes, by 5 cycles, one cycle consisting of reaction at 94°C for 15 seconds and reaction at 72°C for 4 minutes; by

5 cycles, one cycle consisting of reaction at 94°C for 15 seconds and reaction at 70°C for 4 minutes; and by 20 cycles, one cycle consisting of reaction at 94°C for 15 seconds and reaction at 68°C for 4 minutes. Subsequently, PCR was carried out using 50 µl of a reaction solution containing 5 µl of 100-fold dilution of the obtained PCR reaction mixture, 0.2 µmol/l each of the primer having the nucleotide sequence shown in SEQ ID NO: 21 and AP2 primer (attached to the kit), a mixed solution of dNTPs (dATP, dGTP, dCTP and dTTP) containing 200 µmol/l each of the components, 1 µl of a mixed solution of Advantage 2 polymerase and 1 x Advantage 2 PCR buffer under the following conditions.

[0293] That is, using a thermal cycler, PTC-200, PCR was carried out, after heating at 95°C for 3 minutes, by 25 cycles, one cycle consisting of reaction at 94°C for 15 seconds and reaction at 68°C for 3 minutes. A 5 µl aliquot of the resulting PCR reaction mixture was subjected to agarose gel electrophoresis to confirm that an about 0.3 kb DNA fragment was amplified. The DNA fragment was then purified using QIAEX II Gel Extraction Kit (QIAGEN) according to the manual.

[0294] The resulting DNA fragment (50 ng) and 50 ng of T7Blue T-Vector (Novagen) were subjected to ligation using DNA Ligation Kit Ver. 2 (Takara Shuzo) according to the manual to obtain a recombinant plasmid DNA. *Escherichia coli* JM109 was transformed using the recombinant plasmid DNA, and plasmid p432-3 was obtained according to a conventional method.

[0295] The nucleotide sequence of the DNA fragment contained in plasmid p432-3 was determined according to a conventional method, whereby it was found that the DNA fragment inserted could be ligated to plasmid pBF299949. The nucleotide sequence of the DNA fragment inserted is shown in SEQ ID NO: 23. The amino acid sequence of the novel polypeptide encoded by the nucleotide sequence is shown in SEQ ID NO: 22.

[0296] As a result of comparison of the amino acid sequence with that of the human-derived polypeptide of the present invention using an analyzing program [GENETYX WIN ver.2.1 (Software)], 72.6% identity was observed.

[0297] The results of the alignment analysis are shown in Figs. 11 to 13. Fig. 12 is a continuation of Fig. 11 and Fig. 13 is a continuation of Fig. 12.

Example 6

Cloning of a cDNA Fragment of DNA Encoding the Rat-derived Polypeptide of the Present Invention

[0298] Two synthetic primer mixtures were prepared using the information on the amino acid sequence of the human-derived polypeptide of the present invention.

[0299] One of the synthetic primer mixtures is a mixture of primers having the nucleotide sequences in which the bases at positions 3, 6 and 7 are c or t, the bases at positions 9 and 15 are a, c, g or t and the base at position 12 is a or g in the nucleotide sequence shown in SEQ ID NO: 24, and the other is a mixture of primers having the nucleotide sequences in which the base at position 1 is c or t, the base at position 7 is a, c, g or t and the bases at positions 4, 10 and 13 are a or g in the nucleotide sequence shown in SEQ ID NO: 25.

[0300] PCR was carried out using 50 µl of a reaction solution containing 1.0 µmol/l each of the two primer mixtures, 2 µl of cDNA prepared from rat lung-derived mRNA, a mixed solution of dNTPs (dATP, dGTP, dCTP and dTTP) containing 200 µmol/l each of the components, 2.5 units of Taq Gold (Perkin Elmer) and 1 x Taq Gold (Mg plus) buffer (Perkin Elmer) under the following conditions.

[0301] That is, using a thermal cycler, PTC-200 (MJ Research), PCR was carried out, after heating at 95°C for 10 minutes, by 35 cycles, one cycle consisting of reaction at 94°C for one minute and reaction at 60°C for one minute, followed by further heating at 72°C for 8 minutes.

[0302] A 5 µl aliquot of the resulting PCR reaction mixture was subjected to agarose gel electrophoresis to confirm that an anticipated about 0.8 kb DNA fragment was amplified. The DNA fragment was then recovered using QIAEX II Gel Extraction Kit (QIAGEN) according to the manual.

[0303] The DNA fragment recovered above (50 ng) and 50 ng of T7Blue(R)T-Vector (Novagen) were subjected to ligation using DNA Ligation Kit Ver. 2 (Takara Shuzo) according to the manual to obtain a recombinant plasmid DNA. *Escherichia coli* JM109 was transformed using the recombinant plasmid DNA, and plasmid pRp11-2 was obtained according to a conventional method. Determination of the entire nucleotide sequence revealed that plasmid pRp11-2 contained cDNA of about 0.8 kb having the nucleotide sequence shown in SEQ ID NO: 27. The amino acid sequence of the polypeptide encoded by the nucleotide sequence is shown in SEQ ID NO: 26. As a result of comparison of the amino acid sequence with that of the human-derived polypeptide of the present invention using an analyzing program [GENETYX WIN ver. 2.1 (Software)], 72.8% identity was observed. The results of the alignment analysis are shown in Fig. 14.

Example 7

Analysis of Expression Using RT-PCR Method

[0304] A 5'-end DNA primer having the nucleotide sequence shown in SEQ ID NO: 28 and a 3'-end DNA primer having the nucleotide sequence shown in SEQ ID NO: 29 were designed and synthesized based on the nucleotide sequence of the DNA encoding the mouse-derived polypeptide of the present invention determined in Example 5 above. Also, a 5'-end DNA primer having the nucleotide sequence shown in SEQ ID NO: 30 and a 3'-end DNA primer having the nucleotide sequence shown in SEQ ID NO: 31 were designed and synthesized based on the nucleotide sequence of the DNA encoding the rat-derived polypeptide of the present invention determined in Example 6 above. Similarly, a 5'-end DNA primer having the nucleotide sequence shown in SEQ ID NO: 32 and a 3'-end DNA primer having the nucleotide sequence shown in SEQ ID NO: 33 were designed and synthesized based on the information on the nucleotide sequence of mouse cPLA₂α (GenBank NM#008869) to analyze the expression of cPLA₂α. Furthermore, a 5'-end DNA primer having the nucleotide sequence shown in SEQ ID NO: 34 and a 3'-end DNA primer having the nucleotide sequence shown in SEQ ID NO: 35 were designed and synthesized based on the information on the nucleotide sequence of rat cPLA₂ α (GenBank U38376).

[0305] As a control, a 5'-end DNA primer having the nucleotide sequence shown in SEQ ID NO: 36 and a 3'-end DNA primer having the nucleotide sequence shown in SEQ ID NO: 37 were designed and synthesized based on the information on the nucleotide sequences of glyceraldehyde 3-phosphate dehydrogenase (hereinafter referred to as G3PDH) of mouse and rat (GenBank M32599, M17701) to confirm the expression of G3PDH.

[0306] PCR was carried out using 20 μl of a reaction solution containing 0.2 μmol/l each of the combinations of 2 primers (SEQ ID NOS: 28 and 29; SEQ ID NOS: 30 and 31; SEQ ID NOS: 32 and 33; SEQ ID NOS: 34 and 35; and SEQ ID NOS: 36 and 37), 2 μl of cDNA prepared from each of the mRNAs derived from various organs of mouse and rat, a mixed solution of dNTPs (dATP, dGTP, dCTP and dTTP) containing 200 μmol/l each of the components, 2.5 units of Taq Gold polymerase (Perkin Elmer) and 1 x Taq Gold buffer (Mg plus) under the following conditions.

[0307] That is, using a thermal cycler, PTC-200 (MJ Research), PCR was carried out, after heating at 95°C for 10 minutes, by 29 cycles for cPLA₂α and the DNA encoding the polypeptide of the present invention, and by 22 cycles for G3PDH, one cycle consisting of reaction at 94°C for 30 seconds and reaction at 60°C for 30 seconds, followed by further heating at 72°C for 8 minutes.

[0308] A 10 μl aliquot of the resulting PCR reaction mixture was subjected to agarose gel electrophoresis to confirm that an anticipated about 500 bp DNA fragment was amplified. The results of electrophoresis are shown in Fig. 15. A strong expression of the DNA encoding the polypeptide of the present invention was observed in lung and skin.

Example 8Expression of the Mouse-derived Polypeptide of the Present Invention Using an Insect Cell and Measurement of PLA₂ Activity of the Polypeptide

(1) Construction of Plasmid for the Preparation of Baculovirus

[0309] DNA primers having the nucleotide sequences shown in SEQ ID NOS: 40 and 41, respectively, were designed based on the information on the nucleotide sequence of the DNA encoding the mouse-derived polypeptide of the present invention obtained in Example 5 above, and the N-terminal region was amplified by PCR according to the following method.

[0310] That is, PCR was carried out using 50 μl of a reaction solution containing 2 μl of cDNA synthesized from RNA derived from the skin of BALB/C mouse, 0.2 μmol/l each of the primers having the nucleotide sequences shown in SEQ ID NOS: 40 and 41, respectively, a mixed solution of dNTPs (dATP, dGTP, dCTP and dTTP) containing 200 μmol/l each of the components, 1 μl of a mixed solution of Advantage 2 polymerase (Clontech) and 1 x Advantage 2 PCR buffer under the following conditions.

[0311] That is, using a thermal cycler, PTC-200 (MJ Research), PCR was carried out, after heating at 95°C for 2 minutes, by 32 cycles, one cycle consisting of reaction at 94°C for 30 seconds, reaction at 60°C for 30 seconds and reaction at 72°C for 30 seconds, followed by reaction at 72°C for 7 minutes. The PCR reaction mixture was subjected to agarose gel electrophoresis, and an about 1.5 kb DNA fragment was purified using QIAEX II Gel Extraction Kit (QIAGEN) according to the manual.

[0312] The resulting DNA fragment (50 ng) and 50 ng of T7Blue T-Vector (Novagen) were subjected to ligation using DNA Ligation Kit Ver. 2 (Takara Shuzo) according to the manual to obtain a recombinant plasmid DNA. *Escherichia coli* JM109 was transformed using the recombinant plasmid DNA, and plasmid pN3 was obtained according to a conventional method.

[0313] On the other hand, DNA primers having the nucleotide sequences shown in SEQ ID NOS: 42 and 43, respectively, were designed based on the information on the nucleotide sequence, and the C-terminal region was amplified by PCR according to the following method.

[0314] That is, PCR was carried out using 50 µl of a reaction solution containing 2 µl of cDNA synthesized from RNA derived from the skin of BALB/C mouse, 0.2 µmol/l each of the primers having the nucleotide sequences shown in SEQ ID NOS: 42 and 43, respectively, a mixed solution of dNTPs (dATP, dGTP, dCTP and dTTP) containing 200 µmol/l each of the components, 1 µl of a mixed solution of Advantage 2 polymerase (Clontech) and 1 x Advantage 2 PCR buffer under the following conditions.

[0315] That is, using a thermal cycler, PTC-200 (MJ Research), PCR was carried out, after heating at 95°C for 2 minutes, by 32 cycles, one cycle consisting of reaction at 94°C for 30 seconds, reaction at 60°C for 30 seconds and reaction at 72°C for 30 seconds, followed by reaction at 72°C for 7 minutes. The resulting reaction mixture was subjected to extraction with phenol and precipitation with ethanol. The obtained precipitate was dissolved in 50 µl of a buffer consisting of 10 mmol/l Tris-HCl (pH 7.5), 10 mmol/l magnesium chloride and 1 mmol/l DTT, and digestion reaction was carried out at 37°C for 3 hours following the addition of 10 units of Apal and Dral (Takara Shuzo). The resulting reaction mixture was subjected to agarose gel electrophoresis, and an about 1.4 kb DNA fragment was purified using QIAEX II Gel Extraction Kit (QIAGEN).

[0316] Separately, 2 µg of plasmid pBluescript II KS(-) (STRATAGENE) was dissolved in 50 µl of a buffer consisting of 33 mmol/l Tris-acetic acid (pH 7.9), 10 mmol/l magnesium acetate, 0.5 mmol/l DTT, 66 mmol/l potassium acetate and 0.01% BSA, and digestion reaction was carried out at 30°C for 3 hours following the addition of 10 units of SmaI (Takara Shuzo). After extraction with phenol and precipitation with ethanol, the obtained precipitate was dissolved in 30 µl of a buffer consisting of 10 mmol/l Tris-HCl (pH 7.5), 10 mmol/l magnesium chloride and 1 mmol/l DTT, and digesting reaction was carried out at 37°C for 3 hours following the addition of 10 units of Apal (Takara Shuzo). The resulting reaction mixture was subjected to agarose gel electrophoresis, and an about 3.0 kb DNA fragment was purified using QIAEX II Gel Extraction Kit (QIAGEN).

[0317] The PCR-amplified Apal-Dral fragment at C-terminus (1.4 kb) (50 ng) and 50 ng of the plasmid pBluescript II KS(-)-derived SmaI-Apal fragment (3.0 kb) obtained above were subjected to ligation using DNA Ligation Kit Ver. 2 (Takara Shuzo) according to the manual to obtain a recombinant plasmid DNA. *Escherichia coli* JM109 was transformed using the recombinant plasmid DNA, and plasmid pC11 was obtained according to a conventional method.

[0318] The nucleotide sequences of the DNA fragments respectively contained in plasmid pN3 and plasmid pC11 were determined according to a conventional method, and the inserted DNA fragments were subjected to ligation using the SmaI site in each of the inserted fragments under the following conditions.

[0319] That is, 2 µg of plasmid pN3 was dissolved in 50 µl of a buffer consisting of 33 mmol/l Tris-acetic acid (pH 7.9), 10 mmol/l magnesium acetate, 0.5 mmol/l DTT, 66 mmol/l potassium acetate and 0.01% BSA, and digestion reaction was carried out at 30°C for 4 hours following the addition of 10 units of SmaI (Takara Shuzo). The resulting reaction mixture was subjected to agarose gel electrophoresis, and an about 1.3 kb DNA fragment was purified using QIAEX II Gel Extraction Kit (QIAGEN).

[0320] On the other hand, 2 µg of plasmid pC11 was dissolved in 50 µl of a buffer consisting of 33 mmol/l Tris-acetic acid (pH 7.9), 10 mmol/l magnesium acetate, 0.5 mmol/l DTT, 66 mmol/l potassium acetate and 0.01% BSA, and digestion reaction was carried out at 30°C for 4 hours following the addition of 10 units of SmaI (Takara Shuzo). After extraction with phenol and precipitation with ethanol, the obtained precipitate was dissolved in 30 ml of a buffer consisting of 50 mmol/l Tris-HCl (pH 9.0) and 1 mmol/l magnesium chloride, and dephosphorylation reaction was carried out at 60°C for 30 minutes following the addition of 0.5 unit of alkaline phosphatase (Takara Shuzo; derived from *E. coli* C75). The resulting reaction mixture was subjected to agarose gel electrophoresis, and an SmaI-alkaline phosphatase-treated fragment (4.4 kb) was purified using QIAEX II Gel Extraction Kit (QIAGEN).

[0321] The plasmid pN3-derived SmaI fragment (1.3 kb) (50 ng) and the plasmid pC11-derived, SmaI-alkaline phosphatase-treated fragment (4.4 kb) were subjected to ligation using DNA Ligation Kit Ver. 2 (Takara Shuzo) according to the manual to obtain a recombinant plasmid DNA. *Escherichia coli* JM109 was transformed using the recombinant plasmid DNA, and plasmid pN3+C11 was obtained according to a conventional method. The nucleotide sequence resulting from the ligation is shown in SEQ ID NO: 39, and the amino acid sequence of the polypeptide encoded by the nucleotide sequence is shown in SEQ ID NO: 38.

[0322] The amino acid sequence was compared with the human-derived polypeptide of the present invention having the amino acid sequence shown in SEQ ID NO: 1 and the mouse-derived polypeptide of the present invention having the amino acid sequence shown in SEQ ID NO: 22 using an analyzing program [GENETYX WIN ver. 2.1 (Software)]. The results of alignment analysis are shown in Figs. 16 and 17.

[0323] Subsequently, DNA primers having the nucleotide sequences shown in SEQ ID NOS: 44 and 45, respectively, were designed based on the information on the nucleotide sequence, and the N-terminal region into which Flag tag was inserted was amplified by PCR.

[0324] That is, PCR was carried out using 50 µl of a reaction solution containing 10 ng of plasmid pN3, 0.2 µmol/l

each of the primers having the nucleotide sequences shown in SEQ ID NOS: 44 and 45, respectively, a mixed solution of dNTPs (dATP, dGTP, dCTP and dTTP) containing 200 µmol/l each of the components, 1 µl of a mixed solution of Advantage 2 polymerase (Clontech) and 1 x Advantage 2 PCR buffer under the following conditions.

[0325] That is, using a thermal cycler, PTC-200 (MJ Research), PCR was carried out, after heating at 95°C for 2 minutes, by 25 cycles, one cycle consisting of reaction at 94°C for 30 seconds and reaction at 60°C for 30 seconds, followed by reaction at 72°C for 7 minutes. The resulting PCR reaction mixture was subjected to agarose gel electrophoresis, and an about 1.1 kb DNA fragment was purified using QIAEX II Gel Extraction Kit (QIAGEN) according to the manual.

[0326] The obtained DNA fragment (50 ng) and 50 ng of T7Blue T-Vector (Novagen) were subjected to ligation using DNA Ligation Kit Ver. 2 (Takara Shuzo) according to the manual to obtain a recombinant plasmid DNA. *Escherichia coli* JM109 was transformed using the recombinant plasmid DNA, and plasmid pMF11 was obtained according to a conventional method.

[0327] The nucleotide sequence of the DNA fragment contained in plasmid pMF11 was determined according to a conventional method, and the DNA fragment was subjected to ligation with the fragment inserted in plasmid pN3+C11 using *Bst*XI site according to the following method.

[0328] That is, 2 µg of plasmid pMF11 was dissolved in 50 µl of a buffer consisting of 50 mmol/l Tris-HCl (pH 7.5), 10 mmol/l magnesium chloride, 1 mmol/l DTT and 100 mmol/l sodium chloride, and digestion reaction was carried out at 37°C for 3 hours following the addition of 10 units of *Bst*XI (Takara Shuzo). After the reaction mixture was subjected to extraction with phenol and precipitation with ethanol, the obtained precipitate was dissolved in 50 µl of a buffer consisting of 33 mmol/l Tris-acetic acid (pH 7.9), 10 mmol/l magnesium acetate, 0.5 mmol/l DTT, 66 mmol/l potassium acetate and 0.01% BSA, and digestion reaction was carried out at 30°C for 3 hours following the addition of 10 units of *Sma*I (Takara Shuzo). The resulting reaction mixture was subjected to agarose gel electrophoresis, and an about 0.8 kb DNA fragment was purified using QIAEX II Gel Extraction Kit (QIAGEN).

[0329] Plasmid pN3+C11 (2 µg) was dissolved in 50 µl of a buffer consisting of 50 mmol/l Tris-HCl (pH 7.5), 10 mmol/l magnesium chloride, 1 mmol/l DTT and 100 mmol/l sodium chloride, and digestion reaction was carried out at 37°C for 7 hours following the addition of 10 units of *Bst*XI and *Not*I (Takara Shuzo). The resulting reaction mixture was subjected to agarose gel electrophoresis, and an about 1.9 kb DNA fragment was purified using QIAEX II Gel Extraction Kit (QIAGEN).

[0330] On the other hand, 2 µg of plasmid pVL1393 (PharMingen) was dissolved in 50 µl of a buffer consisting of 50 mmol/l Tris-HCl (pH 7.5), 10 mmol/l magnesium chloride, 1 mmol/l DTT and 100 mmol/l sodium chloride, and digestion reaction was carried out at 37°C for 3 hours following the addition of 10 units of *Not*I (Takara Shuzo). After extraction with phenol and precipitation with ethanol, the obtained precipitate was dissolved in 50 µl of a buffer consisting of 33 mmol/l Tris-acetic acid (pH 7.9), 10 mmol/l magnesium acetate, 0.5 mmol/l DTT, 66 mmol/l potassium acetate and 0.01% BSA, and digestion reaction was carried out at 30°C for 3 hours following the addition of 10 units of *Sma*I (Takara Shuzo). The resulting reaction mixture was subjected to agarose gel electrophoresis, and an about 9.6 kb DNA fragment was purified using QIAEX II Gel Extraction Kit (QIAGEN).

[0331] The plasmid pMF11-derived *Sma*I-*Bst*XI fragment (0.8 kb) (50 ng), 50 ng of the plasmid pN3+C11-derived *Bst*XI-*Not*I fragment (1.9 kb) and 50 ng of the pVL1393-derived *Sma*I-*Not*I fragment (9.6 kb) obtained above were subjected to ligation using DNA Ligation Kit Ver. 2 (Takara Shuzo) according to the manual to obtain a recombinant plasmid DNA. *Escherichia coli* JM109 was transformed using the recombinant plasmid DNA, and plasmid pmPLAH-1393 was obtained according to a conventional method. The steps for constructing the plasmid and its restriction map are shown in Fig. 18.

(2) Preparation of Recombinant Baculovirus

[0332] Preparation of a virus was carried out according to the method described in the Baculovirus Expression Vector System Manual (PharMingen).

[0333] That is, 2×10^6 Sf9 cells were seeded on a petri dish of 6 cm in diameter and, after adhesion, the medium was replaced with a serum-free medium (Sf-900IISFM, Life Tech). A mixed solution of DNA and lipofectin (24 µl) containing 5 µg of plasmid pmPLAH-1393 prepared in (1) above or pVL1393, 15 ng of Linearized Baculogold DNA (PharMingen) and 6 ng of lipofectin solution (Life Technologies) was added to the above petri dish containing the serum-free medium so as to distribute evenly, and culturing was carried out at 27°C for 4 days. A medium containing serum (Esf921, Asahi Techno Glass) (2 ml) was added thereto, and culturing was further carried out at 27°C for 3 days. After recovering the cells, the culture was centrifuged at 800 rpm for 5 minutes to obtain a supernatant. The supernatant was added to the Sf9 cells adhered, and culturing was carried out at 27°C for 3 days. After recovering the cells, the culture was centrifuged at 800 rpm for 5 minutes to obtain a supernatant containing a virus.

(3) Preparation of the Soluble Fraction of Insect Cells

[0334] The supernatant containing the virus recovered in (2) above (2 ml) was added to 28 ml of 1.5×10^6 /ml suspending Sf9 cells, and the cells were cultured at 27°C for 4 days in the suspending state. The cells were recovered by centrifugation at 800 rpm for 5 minutes and washed with PBS. The resulting cells were suspended in a buffer consisting of 25 mmol/l Tris-HCl (pH 7.5), 140 mmol/l sodium chloride, 5 mmol/l potassium chloride, 2 mmol/l EDTA and 1 x complete, EDTA-free (Boehringer Mannheim) and disrupted on ice using a sonicator. The extract was centrifuged at 15,000 rpm for 15 minutes, and the supernatant was used for the measurement of PLA₂ activity.

(4) Measurement of PLA₂ Activity

[0335] A reaction solution [100 mmol/l Tris-HCl (pH 7.5), 8 mmol/l calcium chloride, 1 mg/ml BSA (substantially fatty acid-free, Sigma) and 8 μmol/l Triton X-100] (100 μl) containing 2 μmol/l 1-palmitoyl-2-[1-¹⁴C]arachidonylphosphatidylcholine (obtained from Daiichi Kagaku Yakuhin, 48 mCi/mmol) and the supernatant obtained above was incubated at 37°C for 30 minutes, and then Dole reagent (containing 2-propanol, heptane and sulfuric acid at a ratio of 78:20:2) was added thereto to stop the reaction. Calcium concentration dependency was examined at calcium chloride concentrations of 0, 1, 2, 4, 8 and 16 mmol/l, while examination of time dependency was carried out using reaction times of 0, 2, 5, 10, 30, 60 and 90 minutes.

[0336] To the reaction mixture were further added 0.3 ml of heptane and 0.2 ml of water, and mixing was effected by rotation. The resulting mixture was centrifuged at 3,000 rpm for 5 minutes, and 0.32 ml of the obtained upper layer was transferred to a tube containing 40 mg of silica gel (Silica gel 60, Merck), followed by addition of 0.3 ml of heptane. After mixing by rotating the tube, centrifugation was carried out at 3,000 rpm for 5 minutes. A 400 μl aliquot of the supernatant was transferred to a scintillation vial containing 3 ml of Ultima Gold (Packard), and the radioactivity was measured using a liquid scintillation counter (Beckman LS6500). The amount of the polypeptide was determined using the Bio Rad Protein Assay method.

[0337] The soluble fraction of insect cells to which a virus prepared from plasmid pVL1393 had been introduced was used as a control. Figs. 19, 20 and 21 show the results of examination of the dependency on the amount of polypeptide, those on the calcium concentration and those on the reaction time, respectively.

[0338] From the above results, it was revealed that the mouse-derived polypeptide of the present invention obtained in Example 5 above has PLA₂ activity that hydrolyzes the ester bond at the sn-2-position in 1-palmitoyl-2-phosphatidylcholine in a calcium concentration-dependent manner.

Example 9

Analysis of Expression in Cell Lines Using RT-PCR Method

[0339] A 5'-end DNA primer having the nucleotide sequence shown in SEQ ID NO: 46 and a 3'-end DNA primer having the nucleotide sequence shown in SEQ ID NO: 47 were designed and synthesized based on the information on the nucleotide sequence of human cPLA₂α (GenBank ACCESSION M68874).

[0340] PCR amplification of a human cPLA₂α cDNA fragment was carried out using 20 μl of a reaction solution containing 0.2 μmol/l each of the two primers (SEQ ID NOS: 46 and 47), 2 μl of cDNA prepared from each of the RNAs of established human cell lines (K-562, HL-60, Jurkat, 293EBNA, DU145, PC-3 and LNCaP.FGS), a mixed solution of dNTPs (dATP, dGTP, dCTP and dTTP) containing 200 μmol/l each of the components, 2.5 units of Taq Gold polymerase (Perkin Elmer) and 1 x Taq Gold (Mg plus) buffer under the following conditions.

[0341] That is, using a thermal cycler, PTC-200 (MJ Research), PCR was carried out, after heating at 95°C for 10 minutes, by 30 cycles, one cycle consisting of reaction at 94°C for 30 seconds and reaction at 60°C for 30 seconds, followed by heating at 72°C for 8 minutes.

[0342] A 10 μl aliquot of the resulting PCR reaction mixture was subjected to agarose gel electrophoresis to confirm that an anticipated about 0.6 kb DNA fragment was amplified.

[0343] Similarly, PCR amplification of a cDNA fragment of the DNA encoding the human-derived polypeptide of the present invention was carried out. That is, 20 μl of a reaction solution containing 0.2 μmol/l each of the two primers (SEQ ID NOS: 13 and 14), 2 μl of cDNA prepared from each of the RNAs of established human cell lines (K-562, HL-60, Jurkat, 293EBNA, DU145, PC-3 and LNCaP.FGS), a mixed solution of dNTPs (dATP, dGTP, dCTP and dTTP) containing 200 μmol/l each of the components, 2.5 units of Taq Gold polymerase (Perkin Elmer) and 1 x Taq Gold (Mg plus) buffer was used, and PCR amplification was carried out, after heating at 95°C for 10 minutes, by 30 cycles, one cycle consisting of reaction at 94°C for 30 seconds and reaction at 60°C for 30 seconds, followed by heating at 72°C for 8 minutes.

[0344] A 10 μl aliquot of the resulting PCR reaction mixture was subjected to agarose gel electrophoresis to confirm

that an anticipated about 0.6 kb DNA fragment was amplified.

[0345] As a control, PCR amplification of a G3PDH cDNA fragment was carried out. That is, 20 µl of a reaction solution containing 0.2 µmol/l each of the two primers (SEQ ID NOS: 36 and 37), 2 µl of cDNA prepared from each of the RNAs of established human cell lines (K-562, HL-60, Jurkat, 293EBNA, DU145, PC-3 and LNCaP.FGS), a mixed solution of dNTPs (dATP, dGTP, dCTP and dTTP) containing 200 µmol/l each of the components, 2.5 units of Taq Gold polymerase (Perkin Elmer) and 1 x Taq Gold (Mg plus) buffer was used, and PCR amplification was carried out, after heating at 95°C for 10 minutes, by 21 cycles, one cycle consisting of reaction at 94°C for 30 seconds and reaction at 60°C for 30 seconds, followed by heating at 72°C for 8 minutes.

[0346] A 10 µl aliquot of the resulting PCR reaction mixture was subjected to agarose gel electrophoresis to confirm that an anticipated about 0.5 kb DNA fragment was amplified.

[0347] Expression of mRNA for the human-derived polypeptide of the present invention was observed in PC-3 and LNCaP.FGS cells. The results of electrophoresis are shown in Fig. 22.

Example 10

Analysis of Expression of the Human-derived Polypeptide of the Present Invention in Human Fetal Organs by Northern Hybridization

[0348] Northern hybridization was carried out on a poly(A)⁺RNA filter [Human Fetal Normal Tissue mRNA Northern Blot II (Biochain)] of human fetal heart, kidney, skin and small intestine and adult lung in the same manner as in Example 3 using the digoxigenin-labeled cRNA probe prepared in Example 3.

[0349] The results are shown in Fig. 23. Bands of about 3.5 kilo nucleotide and 6 kilo nucleotide were observed in fetal kidney and skin and adult lung.

Industrial Applicability

[0350] The DNA of the novel phospholipase A₂ polypeptide obtained by the present invention is useful for the diagnosis, prevention and treatment of diseases such as asthma, ischemic diseases, arthritis, rheumatism, sepsis, dermatitis, arteriosclerosis, pain, Parkinson disease, Alzheimer disease, malignant tumor, nephritis, diabetes and ischemic reperfusion injury.

SEQUENCE LISTING FREE TEXT

5 SEQ ID NO: 5 - Description of Artificial Sequence:
Synthetic RNA

SEQ ID NO: 6 - Description of Artificial Sequence:
10 Synthetic DNA

SEQ ID NO: 7 - Description of Artificial Sequence:
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SEQ ID NO: 17 - Description of Artificial Sequence:
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SEQ ID NO: 37 - Description of Artificial Sequence:
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SEQ ID NO: 44 - Description of Artificial Sequence:
Synthetic DNA

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Synthetic DNA

55 SEQ ID NO: 46 - Description of Artificial Sequence:
Synthetic DNA

SEQ ID NO: 47 - Description of Artificial Sequence:
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10

15

20

25

30

35

40

45

50

55

EP 1 329 511 A1

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Phe Pro Leu Asn His Gln Asp Ser Gln Glu Leu Gln Val Glu Phe Val
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Leu Glu Lys Ser Gln Val Pro Ala Ser Glu Val Ile Thr Asn Gly Val
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 Glu Lys Ala Phe Gly Asp Phe Val Ile Asn Arg Pro Asp Thr Pro Tyr
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 Gln Lys Arg Glu Lys Arg Gly Pro Leu Trp Arg His Trp Arg Arg Glu
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 Thr Tyr Pro Tyr Tyr Asp Leu Gln Val Lys Val Leu Arg Ala Thr Asn
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 Leu Ala Gly Leu Gln Glu Leu Gly Leu Leu Asp Thr Val Thr Tyr Leu
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 Ser Gly Val Ser Gly Ser Thr Trp Cys Ile Ser Thr Leu Tyr Arg Asp
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50

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gtt cac gtc tgc agc agt aag atg gga gat gtg cgc gtg tct ccc tgc 1409
 Val His Val Cys Ser Ser Lys Met Gly Asp Val Arg Val Ser Pro Cys
 425 430 435

cag tta ccc agg ctt cac tct tgc aac ctg gac cac agt ctc tgg tgatgt 1460
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10 Trp Lys His Arg Arg Glu Thr His Pro Tyr Tyr Asp Leu Gln Val Lys
35 40 45
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15 Asp Cys Tyr Val Arg Leu Trp Leu Pro Thr Ala Ser Val Ser Pro Ser
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Gln Thr Arg Thr Val Val Asn Ser Ser Asp Pro Glu Trp Asn Glu Thr
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30 Lys Ser Gln Thr Pro Ala Ser Glu Val Val Thr Asn Gly Val Leu Val
165 170 175
Ala His Pro Cys Leu Arg Ile Gln Gly Thr Val Thr Gly Asp Lys Thr
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45 Ser Asp Glu Leu Glu Ala Gln Thr Ser Lys Met Asp Lys Ala Ser Ile
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50 Leu Glu Glu Gly Gln Gln Val Thr Leu Arg Met Lys Ala Asp Met Ser
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Ser Ser Gly Asp Leu Asp Leu Arg Leu Gly Phe Asp Leu Cys Asp Gly
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			595				600						605				
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		610					615					620					
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55	Met	Tyr	Lys	Asp	Tyr	Thr	Ala	Arg	Lys	Asp	Phe	Val	Val	Ser	Glu	Asp	

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	Leu Thr Pro Met Lys Asp Phe Leu Ser Leu Val Asp Gly Gly Phe Ala 675 680 685		
10	Ile Asn Ser Pro Phe Pro Leu Val Leu Gln Pro Gln Arg Ala Val Asp 690 695 700		
	Leu Ile Val Ser Phe Asp Tyr Ser Leu Glu Gly Pro Phe Glu Val Leu 705 710 715 720		
15	Gln Val Thr Glu Lys Tyr Cys Arg Asp Arg Gly Ile Pro Phe Pro Arg 725 730 735		
	Ile Glu Val Asp Pro Lys Asp Ser Glu Asp Pro Arg Glu Cys Tyr Leu 740 745 750		
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	Leu Val Asn Arg Thr Phe Arg Thr His Leu Ala Pro Gly Val Glu Arg 770 775 780		
25	Gln Thr Ala Glu Glu Lys Ala Phe Gly Asp Phe Ile Ile Asn Gly Pro 785 790 795 800		
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30	Asp Arg Leu Val Thr Leu Ser Arg Tyr Asn Val Leu Asn Asn Lys Glu 820 825 830		
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	Pro Gln Trp Lys His Arg Arg Glu Thr His Pro Tyr Tyr Asp Leu Gln	
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	Val Lys Val Leu Arg Ala Arg Asn Ile Gln His Thr Asp Lys Leu Ser	
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15	aaa gcc gac tgc tat gtt cga ctg tgg ctg ccc acg gct tct gtt agc	302
	Lys Ala Asp Cys Tyr Val Arg Leu Trp Leu Pro Thr Ala Ser Val Ser	
	65 70 75	
20	ccc agt cag aca agg aca gtg gtt aac agc agt gat cca gaa tgg aat	350
	Pro Ser Gln Thr Arg Thr Val Asn Ser Ser Asp Pro Glu Trp Asn	
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25	gag acc ttt cac tat cag atc cac ggc gct gtg aag aac gtc ttg gag	398
	Glu Thr Phe His Tyr Gln Ile His Gly Ala Val Lys Asn Val Leu Glu	
	95 100 105 110	
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	Leu Ala Leu Tyr Asp Glu Asp Val Leu Asp Ser Asp Asn Val Phe Ser	
	115 120 125	
35	att ctg ttt gac atg agt act ctc cag cta ggc cag cct tgc aca aaa	494
	Ile Leu Phe Asp Met Ser Thr Leu Gln Leu Gly Gln Pro Cys Thr Lys	
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40	aac ttc acc agg cag cag gat cca aag gag ctg gaa gta gaa ttt act	542
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	Leu Glu Lys Ser Gln Thr Pro Ala Ser Glu Val Val Thr Asn Gly Val	
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	Leu Val Ala His Pro Cys Leu Arg Ile Gln Gly Thr Val Thr Gly Asp	
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	Pro Gly Leu Pro Val Asn Phe Thr Phe His Met Asn Pro Val Leu Ser	
	225 230 235	
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	Pro Lys Leu His Ile Lys Leu Gln Glu Gln Leu Gln Val Phe His Ser	
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	Gly Pro Ser Asp Glu Leu Glu Ala Gln Thr Ser Lys Met Asp Lys Ala	
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	Val	Pro	Val	Val	Ala	Val	Leu	Gly	Ser	Gly	Gly	Gly	Thr	Arg	Ala	Met	
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	Asp	Ala	Val	Thr	Tyr	Leu	Ser	Gly	Val	Ser	Gly	Ser	Ser	Trp	Cys	Ile	
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	Pro	Ile	Lys	Tyr	Ala	Ser	Glu	Arg	Val	Cys	Ser	Ser	Lys	Ile	Gly	Met	
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20	cta agc ctg agc ttt ctg gac tgg cac agg ggg agt gtc agt gtc aca Leu Ser Leu Ser Phe Leu Asp Trp His Arg Gly Ser Val Ser Val Thr 575 580 585 590	1838		
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20 gaa ttt gat cgg ctg gtg acc ctg agc cga tac aac gtc ttg aac aac 2558
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40 ccaggcagca gcttcacag aaggcgcacc cccaccccca ccacagaagg acctctggaa 2900
 agaacccaag cattcagagc ttcaccacag agctgtgggc tgaggaacca gctgtcctta 2960
 cactgatgca gaactacagc tgctcacact tccacagagt ggccagctct gacccactcc 3020

45 aagccccgg actcagtgat gtggagaata aacagcagct atgtgggtcg ccagcctgtg 3080
 tcactgaaaa aaaaaaaaaa aaaaaaaaaa aa 3112

50 <210> 24
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 <212> DNA
 <213> Artificial Sequence

55 <220>
 <223> Description of Artificial Sequence: Synthetic DNA

60 <400> 24
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65 <210> 25
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70 <220>
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<400> 25
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5 <210> 26
<211> 261
<212> PRT
<213> Rattus norvegicus

10 <400> 26
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1 5 10 15

Ile Phe Leu Lys Met Arg Gly Pro Arg Leu Gly Phe Leu Asp Trp His
20 25 30

15 Arg Gly Thr Val Ser Val Thr Asp Asp Trp Pro Lys Leu Arg Lys Gln
35 40 45

Asp Pro Thr Arg Leu Pro Thr Arg Leu Phe Thr Ser Lys Ser Phe Phe
50 55 60

20 Ser Lys Ala Val Leu Asp Ile Phe Thr Ser Arg Phe Thr Cys Ala Gln
65 70 75 80

Thr Phe Asn Phe Thr Arg Gly Leu Cys Leu Tyr Lys Asp Tyr Thr Ala
85 90 95

25 Arg Lys Asp Phe Val Val Ser Glu Asp Ala Trp His Ser Asp Asn Tyr
100 105 110

Lys His Leu Asp Ala Cys Pro Asn Gln Leu Thr Pro Met Lys Asp Phe
115 120 125

30 Leu Ser Leu Val Asp Gly Gly Phe Ala Ile Asn Ser Pro Phe Pro Leu
130 135 140

Ile Leu Gln Pro Gln Arg Ala Val Asp Leu Ile Val Ser Phe Asp Tyr
145 150 155 160

Ser Leu Glu Ala Pro Phe Glu Val Leu Gln Val Thr Glu Lys Tyr Cys
165 170 175

35 Arg Asp Arg Gly Ile Pro Phe Pro Arg Ile Glu Val Asp Pro Lys Asp
180 185 190

Ser Lys Asp Pro Arg Glu Cys Tyr Leu Phe Thr Glu Ala Glu Asp Pro
195 200 205

40 Cys Ser Pro Ile Val Leu His Phe Pro Leu Val Asn Arg Thr Phe Arg
210 215 220

Lys His Leu Ala Pro Gly Val Glu Arg Gln Thr Ala Glu Glu Lys Ala
225 230 235 240

50 Phe Gly Asp Phe Ile Ile Asn Gly Pro Asp Thr Ala Tyr Gly Met Met
245 250 255

Asn Phe Thr Tyr Glu
260

55 <210> 27

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<211> 783
<212> DNA
<213> Rattus norvegicus

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10
15
20
25
30
gag 783

<400> 27
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atgagaggcc caagactggg cttcctggac tggcacagag gcactgtcag tgtcacagat 120
gactggccaa agttacggaa gcaggacccc actcggctgc ccaccaggct ctttacctca 180
aagagtttct tctctaaggc tgtgctggac atattcacct cccgctttac ttgtgcccag 240
acctttaact ttaccgagg tctctgcctg tacaaggact acacagctag aaaggacttt 300
gtggctctctg aagatgcatg gcattcagat aattacaaac acctcgatgc ctgtcccaac 360
cagcttacac ccatgaagga cttcctgtcc ttagtggatg gaggctttgc catcaactca 420
ccattccac tgatcctgca gcgcagcgg gctgtggacc tcattgtgtc ctttgactat 480
tccttggaag ccccttttga ggtcctgcag gtgacagaga agtactgccg ggaccgaggg 540
atccccctcc caaggattga ggtagacccc aaggactcta aggacccccg tgaatgctat 600
ctgtttactg aggcgaggga cccctgctcg cccattgtgc tgcattttcc tcttgtcaac 660
aggacctttc gcaaacacct gggtccagga gtggaacgac aaacagctga ggagaaggcc 720
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<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic DNA

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<210> 29
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic DNA

<400> 29
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<210> 30
<211> 21
<212> DNA
<213> Artificial Sequence

<220>

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<223> Description of Artificial Sequence: Synthetic DNA

5   <400> 30
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    <212> DNA
10  <213> Artificial Sequence

    <220>
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    <400> 31
15  aaggccttct cctcagctgt                               20

    <210> 32
    <211> 21
    <212> DNA
20  <213> Artificial Sequence

    <220>
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    <210> 33
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    <212> DNA
30  <213> Artificial Sequence

    <220>
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    <210> 34
    <211> 21
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40  <213> Artificial Sequence

    <220>
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    <210> 35
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50  <213> Artificial Sequence

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    <400> 35
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    <210> 36

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 <210> 37
 <211> 26
 <212> DNA
 <213> Artificial Sequence
 15
 <220>
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 <400> 37
 20 ttcaccacct tcttgatgtc atcata 26
 <210> 38
 <211> 853
 <212> PRT
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 25
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 1 5 10 15
 30 Leu Leu Gly Ala Ile Leu Leu Arg Lys Thr Glu Lys Ser Glu Pro Gln
 20 25 30
 Trp Lys His Arg Arg Glu Thr His Pro Tyr Tyr Asp Leu Gln Val Lys
 35 40 45
 35 Val Leu Arg Ala Arg Asn Ile Gln His Thr Asp Lys Leu Ser Lys Ala
 50 55 60
 Asp Cys Tyr Val Arg Leu Trp Leu Pro Thr Ala Ser Val Ser Pro Ser
 65 70 75 80
 40 Gln Thr Arg Thr Val Val Asn Ser Ser Asp Pro Glu Trp Asn Glu Thr
 85 90 95
 Phe Pro Tyr Gln Ile His Gly Ala Val Lys Asn Val Leu Glu Leu Ala
 100 105 110
 45 Leu Tyr Asp Glu Asp Val Leu Asp Ser Asp Asn Val Phe Ser Ile Leu
 115 120 125
 Phe Asp Thr Ser Thr Leu Gln Leu Gly Gln Pro Cys Thr Lys Asn Phe
 130 135 140
 50 Thr Arg Gln Gln Asp Pro Lys Glu Leu Glu Val Glu Phe Thr Leu Glu
 145 150 155 160
 Lys Ser Gln Thr Pro Ala Ser Glu Val Val Thr Asn Gly Val Leu Val
 165 170 175
 55 Ala His Pro Cys Leu Arg Ile Gln Gly Thr Val Thr Gly Asp Lys Thr

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	180	185	190
5	Ala Ser Leu Gly Glu Leu Gly Ser Arg Gln Ile Gln Leu Ala Val Pro 195 200 205		
	Gly Ala Tyr Glu Lys Pro Gln Pro Leu Gln Pro Thr Ser Glu Pro Gly 210 215 220		
10	Leu Pro Val Asn Phe Thr Phe His Val Asn Pro Val Leu Ser Pro Lys 225 230 235 240		
	Leu His Ile Lys Leu Gln Glu Gln Leu Gln Val Phe His Ser Gly Pro 245 250 255		
15	Ser Asp Glu Leu Glu Ala Gln Thr Ser Lys Met Asp Lys Ala Ser Ile 260 265 270		
	Leu Leu Ser Ser Leu Pro Leu Asn Glu Glu Leu Thr Lys Leu Val Asp 275 280 285		
20	Leu Glu Glu Gly Gln Gln Val Ser Leu Arg Met Lys Ala Asp Met Ser 290 295 300		
	Ser Gly Asp Leu Asp Leu Arg Leu Gly Phe Asp Leu Cys Asp Gly Glu 305 310 315 320		
25	Gln Glu Phe Leu Asp Lys Arg Lys Gln Val Ala Ser Lys Ala Leu Gln 325 330 335		
	Arg Val Met Gly Leu Ser Glu Ala Leu His Cys Asp Gln Val Pro Val 340 345 350		
30	Val Ala Val Leu Gly Ser Gly Gly Gly Thr Arg Ala Met Thr Ser Leu 355 360 365		
	Tyr Gly Ser Leu Ala Gly Leu Gln Glu Leu Gly Leu Leu Asp Ala Val 370 375 380		
35	Thr Tyr Leu Ser Gly Val Ser Gly Ser Ser Trp Cys Ile Ser Thr Leu 385 390 395 400		
	Tyr Arg Asp Pro Ser Trp Ser Gln Lys Ala Leu Gln Gly Pro Ile Lys 405 410 415		
40	Tyr Ala Ser Glu Arg Val Cys Ser Ser Lys Ile Gly Met Leu Ser Pro 420 425 430		
	Lys Gln Phe Glu Tyr Tyr Ser Arg Glu Lys Arg Ala Trp Glu Ser Arg 435 440 445		
45	Gly His Ser Met Ser Phe Thr Asp Leu Trp Gly Leu Ile Ile Glu Tyr 450 455 460		
50	Phe Leu Asn Gln Glu Glu Asn Pro Ala Lys Leu Ser Asp Gln Gln Glu 465 470 475 480		
	Thr Val Ser Gln Gly Gln Asn Pro Tyr Pro Ile Tyr Ala Ser Ile Asn 485 490 495		
55	Val His Lys Asn Ile Ser Gly Asp Asp Phe Ala Glu Trp Cys Glu Phe 500 505 510		

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5	Thr	Pro	Tyr	Glu	Val	Gly	Phe	Pro	Lys	Tyr	Gly	Ala	Tyr	Val	Pro	Thr	515	520	525
	Glu	Leu	Phe	Gly	Ser	Glu	Phe	Phe	Met	Gly	Arg	Leu	Leu	His	Phe	Trp	530	535	540
10	Pro	Glu	Pro	Arg	Ile	Cys	Tyr	Leu	Gln	Gly	Met	Trp	Gly	Ser	Ala	Phe	545	550	555
	Ala	Ala	Ser	Leu	Tyr	Glu	Ile	Phe	Leu	Lys	Leu	Gly	Gly	Leu	Ser	Leu	565	570	575
15	Ser	Phe	Leu	Asp	Trp	His	Arg	Gly	Ser	Val	Ser	Val	Thr	Asp	Asp	Trp	580	585	590
	Pro	Lys	Leu	Arg	Lys	Gln	Asp	Pro	Thr	Arg	Leu	Pro	Thr	Arg	Leu	Phe	595	600	605
20	Thr	Pro	Met	Ser	Ser	Phe	Ser	Gln	Ala	Val	Leu	Asp	Ile	Phe	Thr	Ser	610	615	620
	Arg	Ile	Thr	Cys	Ala	Gln	Thr	Phe	Asn	Phe	Thr	Arg	Gly	Leu	Cys	Met	625	630	635
25	Tyr	Lys	Asp	Tyr	Thr	Ala	Arg	Lys	Asp	Phe	Val	Val	Ser	Glu	Asp	Ala	645	650	655
	Trp	His	Ser	His	Asn	Tyr	Gly	Tyr	Pro	Asp	Ala	Cys	Pro	Asn	Gln	Leu	660	665	670
30	Thr	Pro	Met	Lys	Asp	Phe	Leu	Ser	Leu	Val	Asp	Gly	Gly	Phe	Ala	Ile	675	680	685
	Asn	Ser	Pro	Phe	Pro	Leu	Val	Leu	Gln	Pro	Gln	Arg	Ala	Val	Asp	Leu	690	695	700
35	Ile	Val	Ser	Phe	Asp	Tyr	Ser	Leu	Glu	Gly	Pro	Phe	Glu	Val	Leu	Gln	705	710	715
	Val	Thr	Glu	Lys	Tyr	Cys	Arg	Asp	Arg	Gly	Ile	Pro	Phe	Pro	Arg	Ile	725	730	735
40	Glu	Val	Asp	Pro	Lys	Asp	Ser	Glu	Asp	Pro	Arg	Glu	Cys	Tyr	Leu	Phe	740	745	750
	Ala	Glu	Ala	Glu	Asp	Pro	Cys	Ser	Pro	Ile	Val	Leu	His	Phe	Pro	Leu	755	760	765
45	Val	Asn	Arg	Thr	Phe	Arg	Thr	His	Leu	Ala	Pro	Gly	Val	Glu	Arg	Gln	770	775	780
	Thr	Ala	Glu	Glu	Lys	Ala	Phe	Gly	Asp	Phe	Ile	Ile	Asn	Gly	Pro	Asp	785	790	795
50	Thr	Ala	Tyr	Gly	Met	Met	Asp	Phe	Thr	Tyr	Glu	Pro	Lys	Glu	Phe	Asp	805	810	815
	Arg	Leu	Val	Thr	Leu	Ser	Arg	Tyr	Asn	Val	Leu	Asn	Asn	Lys	Glu	Thr	820	825	830

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Ile Arg His Ala Leu Gln Leu Ala Leu Asp Arg Arg Arg Gln Ala Gly
835 840 845

5 Gly Arg Val Gly Gly
850

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<220>
15 <221> CDS
<222> (52)...(2610)

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Met Pro
1

20 tgg act ctc cag cca aag tgg ctg gca ggc aag gga ctt ccc ctt ctt 105
Trp Thr Leu Gln Pro Lys Trp Leu Ala Gly Lys Gly Leu Pro Leu Leu
5 10 15

25 gga gcc ata ctg cta cgg aag aca gaa aag agc gaa cca caa tgg aag 153
Gly Ala Ile Leu Leu Arg Lys Thr Glu Lys Ser Glu Pro Gln Trp Lys
20 25 30

30 cat agg cgg gaa acc cac cca tac tac gac ctt caa gtg aag gtg ctg 201
His Arg Arg Glu Thr His Pro Tyr Tyr Asp Leu Gln Val Lys Val Leu
35 40 45 50

agg gcc aga aac atc cag cac aca gat aag ttg tcc aaa gcc gac tgc 249
Arg Ala Arg Asn Ile Gln His Thr Asp Lys Leu Ser Lys Ala Asp Cys
55 60 65

35 tat gtt cga ctg tgg ctg ccc acg gct tct gtt agc ccc agt cag aca 297
Tyr Val Arg Leu Trp Leu Pro Thr Ala Ser Val Ser Pro Ser Gln Thr
70 75 80

40 agg aca gtg gtt aac agc agt gat cca gaa tgg aat gag acc ttt ccc 345
Arg Thr Val Val Asn Ser Ser Asp Pro Glu Trp Asn Glu Thr Phe Pro
85 90 95

45 tat cag atc cac ggc gct gtg aag aac gtc ctg gag ctt gcc ctt tat 393
Tyr Gln Ile His Gly Ala Val Lys Asn Val Leu Glu Leu Ala Leu Tyr
100 105 110

gac gag gat gtc ctg gac agt gac aat gtc ttc tcc att ctg ttt gac 441
Asp Glu Asp Val Leu Asp Ser Asp Asn Val Phe Ser Ile Leu Phe Asp
115 120 125 130

50 acg agt act ctt cag cta ggc cag cct tgc aca aaa aac ttc acc agg 489
Thr Ser Thr Leu Gln Leu Gly Gln Pro Cys Thr Lys Asn Phe Thr Arg
135 140 145

cag cag gat cca aaa gag ctg gaa gta gaa ttt act ctg gaa aag agt 537
Gln Gln Asp Pro Lys Glu Leu Glu Val Glu Phe Thr Leu Glu Lys Ser
150 155 160

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10	ccc tgt ctg aga att cag ggc aca gtc act gga gac aag aca gcc tcc Pro Cys Leu Arg Ile Gln Gly Thr Val Thr Gly Asp Lys Thr Ala Ser 180 185 190	633
15	ctt gga gag ttg ggc tcc agg cag atc cag ctg gca gtg cct ggg gcc Leu Gly Glu Leu Gly Ser Arg Gln Ile Gln Leu Ala Val Pro Gly Ala 195 200 205 210	681
20	tat gaa aag cca cag cct ctg cag cca acc tcg gag cca ggc ctc cca Tyr Glu Lys Pro Gln Pro Leu Gln Pro Thr Ser Glu Pro Gly Leu Pro 215 220 225	729
25	gtg aac ttt acc ttc cac gtg aac cca gtg ctg agc ccc aag ctg cac Val Asn Phe Thr Phe His Val Asn Pro Val Leu Ser Pro Lys Leu His 230 235 240	777
30	ata aag ctg caa gaa cag ctc caa gtc ttc cat agt ggc ccg agt gat Ile Lys Leu Gln Glu Gln Leu Gln Val Phe His Ser Gly Pro Ser Asp 245 250 255	825
35	gag ctg gaa gct cag acc agc aag atg gac aag gca agc atc ctg ctc Glu Leu Glu Ala Gln Thr Ser Lys Met Asp Lys Ala Ser Ile Leu Leu 260 265 270	873
40	tcc tct ctg ccc ctc aac gag gag tta acg aaa ctt gtg gac ctg gag Ser Ser Leu Pro Leu Asn Glu Glu Leu Thr Lys Leu Val Asp Leu Glu 275 280 285 290	921
45	gag ggc cag cag gtg tct ctt agg atg aag gca gac atg agc tct ggg Glu Gly Gln Gln Val Ser Leu Arg Met Lys Ala Asp Met Ser Ser Gly 295 300 305	969
50	gac ttg gac ctg cgc ctt ggt ttt gac ctc tgt gat gga gag cag gaa Asp Leu Asp Leu Arg Leu Gly Phe Asp Leu Cys Asp Gly Glu Gln Glu 310 315 320	1017
55	ttt ctg gac aag agg aag cag gtg gcg tcc aag gcc ctg cag cgg gtg Phe Leu Asp Lys Arg Lys Gln Val Ala Ser Lys Ala Leu Gln Arg Val 325 330 335	1065
60	atg gga ttg agt gag gct ctg cac tgt gac cag gta cct gtg gta gcc Met Gly Leu Ser Glu Ala Leu His Cys Asp Gln Val Pro Val Val Ala 340 345 350	1113
65	gtg tta ggc tct ggg ggt gga acc aga gcc atg act tcc ctg tac ggc Val Leu Gly Ser Gly Gly Gly Thr Arg Ala Met Thr Ser Leu Tyr Gly 355 360 365 370	1161
70	agc ctg gct ggg ctg cag gag ctt ggt ctt ctg gat gcc gtg acc tac Ser Leu Ala Gly Leu Gln Glu Leu Gly Leu Leu Asp Ala Val Thr Tyr 375 380 385	1209
75	ctg agt ggg gtc tct ggg tct tcc tgg tgc atc tct aca ctc tac agg Leu Ser Gly Val Ser Gly Ser Ser Trp Cys Ile Ser Thr Leu Tyr Arg 390 395 400	1257
80	gat cca tcc tgg tcc cag aag gct ttg cag ggc ccc att aaa tat gcc	1305

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5	tca	gag	cga	gtc	tgc	agc	agt	aaa	att	ggg	atg	ctg	tcc	cca	aag	cag	1353
	Ser	Glu	Arg	Val	Cys	Ser	Ser	Lys	Ile	Gly	Met	Leu	Ser	Pro	Lys	Gln	
			420					425				430					
10	ttt	gaa	tac	tac	tcc	cgg	gaa	aag	aga	gcc	tgg	gag	agc	agg	gga	cac	1401
	Phe	Glu	Tyr	Tyr	Ser	Arg	Glu	Lys	Arg	Ala	Trp	Glu	Ser	Arg	Gly	His	
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15	agc	atg	tcc	ttc	act	gac	ttg	tgg	ggc	ctc	atc	att	gag	tat	ttc	ctg	1449
	Ser	Met	Ser	Phe	Thr	Asp	Leu	Trp	Gly	Leu	Ile	Ile	Glu	Tyr	Phe	Leu	
					455					460					465		
20	aac	cag	gag	gaa	aac	cct	gcc	aag	ctg	tca	gac	cag	caa	gaa	acg	gtc	1497
	Asn	Gln	Glu	Glu	Asn	Pro	Ala	Lys	Leu	Ser	Asp	Gln	Gln	Glu	Thr	Val	
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	Ser	Gln	Gly	Gln	Asn	Pro	Tyr	Pro	Ile	Tyr	Ala	Ser	Ile	Asn	Val	His	
			485					490					495				
30	aaa	aac	atc	agt	ggg	gac	gac	ttt	gca	gag	tgg	tgc	gag	ttc	acc	ccc	1593
	Lys	Asn	Ile	Ser	Gly	Asp	Asp	Phe	Ala	Glu	Trp	Cys	Glu	Phe	Thr	Pro	
			500				505					510					
35	tat	gag	gtc	ggg	ttc	ccc	aag	tac	ggg	gct	tac	ggt	ccc	acg	gaa	ctc	1641
	Tyr	Glu	Val	Gly	Phe	Pro	Lys	Tyr	Gly	Ala	Tyr	Val	Pro	Thr	Glu	Leu	
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40	ttt	ggc	tct	gaa	ttc	ttc	atg	ggc	cgg	ctg	ctg	cat	ttc	tgg	cca	gag	1689
	Phe	Gly	Ser	Glu	Phe	Phe	Met	Gly	Arg	Leu	Leu	His	Phe	Trp	Pro	Glu	
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45	ccc	cgc	atc	tgt	tac	ctg	cag	ggg	atg	tgg	gga	agt	gct	ttt	gca	gcc	1737
	Pro	Arg	Ile	Cys	Tyr	Leu	Gln	Gly	Met	Trp	Gly	Ser	Ala	Phe	Ala	Ala	
				550					555					560			
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	Ser	Leu	Tyr	Glu	Ile	Phe	Leu	Lys	Leu	Gly	Gly	Leu	Ser	Leu	Ser	Phe	
			565					570					575				
55	ctg	gac	tgg	cac	agg	ggg	agt	gtc	agt	gtc	aca	gat	gac	tgg	cca	aag	1833
	Leu	Asp	Trp	His	Arg	Gly	Ser	Val	Ser	Val	Thr	Asp	Asp	Trp	Pro	Lys	
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60	tta	cgg	aag	cag	gac	ccc	aca	cgg	ctg	cct	acc	aga	ctc	ttc	acg	cca	1881
	Leu	Arg	Lys	Gln	Asp	Pro	Thr	Arg	Leu	Pro	Thr	Arg	Leu	Phe	Thr	Pro	
						600					605					610	
65	atg	agt	tcc	ttc	tct	cag	gct	gtg	ctg	gac	ata	ttc	acc	tcc	cgt	att	1929
	Met	Ser	Ser	Phe	Ser	Gln	Ala	Val	Leu	Asp	Ile	Phe	Thr	Ser	Arg	Ile	
					615					620					625		
70	act	tgt	gcc	cag	acc	ttt	aac	ttt	acc	cga	ggg	ctc	tgc	atg	tac	aaa	1977
	Thr	Cys	Ala	Gln	Thr	Phe	Asn	Phe	Thr	Arg	Gly	Leu	Cys	Met	Tyr	Lys	
				630					635					640			
75	gac	tac	aca	gct	aga	aag	gac	ttc	gtg	gtc	tct	gaa	gat	gca	tgg	cat	2025
	Asp	Tyr	Thr	Ala	Arg	Lys	Asp	Phe	Val	Val	Ser	Glu	Asp	Ala	Trp	His	

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5	tca cat aac tat gga tac cct gat gcc tgt ccc aac cag ctc aca ccc Ser His Asn Tyr Gly Tyr Pro Asp Ala Cys Pro Asn Gln Leu Thr Pro 660 665 670	2073		
10	atg aag gac ttc ctg tcc cta gta gat gga ggc ttt gct atc aac tcg Met Lys Asp Phe Leu Ser Leu Val Asp Gly Gly Phe Ala Ile Asn Ser 675 680 685 690	2121		
15	cca ttt cca ctg gtc ctg cag ccg cag cgg gct gtg gac ctc att gtg Pro Phe Pro Leu Val Leu Gln Pro Gln Arg Ala Val Asp Leu Ile Val 695 700 705	2169		
20	tcc ttt gac tat tcc ttg gaa ggc cct ttt gag gtc ctg cag gtg aca Ser Phe Asp Tyr Ser Leu Glu Gly Pro Phe Glu Val Leu Gln Val Thr 710 715 720	2217		
25	gag aag tac tgc cgg gac cga ggg atc ccc ttc cca agg att gag gtg Glu Lys Tyr Cys Arg Asp Arg Gly Ile Pro Phe Pro Arg Ile Glu Val 725 730 735	2265		
30	gac ccc aag gac tct gaa gac ccc cgt gaa tgc tat ctg ttt gct gag Asp Pro Lys Asp Ser Glu Asp Pro Arg Glu Cys Tyr Leu Phe Ala Glu 740 745 750	2313		
35	gca gag gac ccc tgc tgc ccc atc gtg ctg cat ttc cct ctt gtc aac Ala Glu Asp Pro Cys Ser Pro Ile Val Leu His Phe Pro Leu Val Asn 755 760 765 770	2361		
40	agg acc ttt cgc acg cac ctg gcc cca ggt gtg gaa cga caa aca gct Arg Thr Phe Arg Thr His Leu Ala Pro Gly Val Glu Arg Gln Thr Ala 775 780 785	2409		
45	gag gag aag gcc ttc ggg gac ttt atc atc aac ggg cca gat act gcc Glu Glu Lys Ala Phe Gly Asp Phe Ile Asn Gly Pro Asp Thr Ala 790 795 800	2457		
50	tat ggc atg atg gat ttc acc tac gag ccc aag gaa ttt gat cgg ctg Tyr Gly Met Met Asp Phe Thr Tyr Glu Pro Lys Glu Phe Asp Arg Leu 805 810 815	2505		
55	gtg acc ctg agc cga tac aac gtc ttg aac aac aag gag act atc agg Val Thr Leu Ser Arg Tyr Asn Val Leu Asn Asn Lys Glu Thr Ile Arg 820 825 830	2553		
60	cat gcc ctc cag ctg gct ctg gac cgg cgg cgg cag gct ggg gga agg His Ala Leu Gln Leu Ala Leu Asp Arg Arg Arg Gln Ala Gly Gly Arg 835 840 845 850	2601		
65	gtt ggg ggc tgatcacatg agagtcagag gactgtggtg gtgtgatgga Val Gly Gly	2650		
70	ggaccttaag tcagagtatg ctgagggaga gggaagactt taaa	2694		
75	<210> 40 <211> 20 <212> DNA <213> Artificial Sequence			
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EP 1 329 511 A1

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<220>
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<220>
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<210> 47
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<220>
 <223> Description of Artificial Sequence: Synthetic DNA

<400> 47
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Claims

1. A polypeptide having an amino acid sequence selected from the group consisting of the amino acid sequences shown in SEQ ID NOS: 1, 22, 26 and 38.
2. A polypeptide consisting of an amino acid sequence wherein one or more amino acid residues are deleted, substituted or added in an amino acid sequence selected from the group consisting of the amino acid sequences shown in SEQ ID NOS: 1, 22, 26 and 38 and having phospholipase A₂ activity.
3. A polypeptide consisting of an amino acid sequence which has 60% or more homology to an amino acid sequence selected from the group consisting of the amino acid sequences shown in SEQ ID NOS: 1, 22, 26 and 38 and having phospholipase A₂ activity.
4. A DNA encoding the polypeptide according to any of Claims 1 to 3.
5. A DNA having a nucleotide sequence selected from the group consisting of the nucleotide sequences shown in SEQ ID NOS: 2, 23, 27 and 39.
6. A DNA which hybridizes to a DNA consisting of a nucleotide sequence complementary to a nucleotide sequence selected from the group consisting of the nucleotide sequences shown in SEQ ID NOS: 2, 23, 27 and 39 under stringent conditions and which encodes a polypeptide having phospholipase A₂ activity.
7. A recombinant vector comprising the DNA according to any of Claims 4 to 6.
8. A transformant carrying the recombinant vector according to Claim 7.
9. The transformant according to Claim 8, wherein the transformant is selected from the group consisting of a microorganism, an animal cell, a plant cell and an insect cell.
10. The transformant according to Claim 9, wherein the microorganism is a microorganism belonging to the genus Escherichia.

11. The transformant according to Claim 9, wherein the microorganism is Escherichia coli JM109/p5269+C5 (FERM BP-7281).
- 5 12. A process for producing a polypeptide having phospholipase A₂ activity, which comprises culturing the transformant according to any of Claims 8 to 11 in a medium, allowing the polypeptide having phospholipase A₂ activity to form and accumulate in the culture, and recovering the polypeptide from the culture.
- 10 13. An oligonucleotide selected from the group consisting of a sense oligonucleotide having a nucleotide sequence identical with a sequence of 5 to 60 consecutive nucleotides in the nucleotide sequence of the DNA according to any of Claims 4 to 6, an antisense oligonucleotide having a nucleotide sequence complementary to that of said sense oligonucleotide, and a derivative of said sense oligonucleotide or antisense oligonucleotide.
- 15 14. An oligonucleotide consisting of a nucleotide sequence selected from the group consisting of the nucleotide sequences shown in SEQ ID NOS: 13, 14, 28, 29, 30, 31, 46 and 47.
- 20 15. The oligonucleotide according to Claim 13, wherein the oligonucleotide derivative is selected from the group consisting of an oligonucleotide derivative wherein the phosphodiester bond in an oligonucleotide is converted to a phosphorothioate bond, an oligonucleotide derivative wherein the phosphodiester bond in an oligonucleotide is converted to an N3'-P5' phosphoamidate bond, an oligonucleotide derivative wherein the ribose-phosphodiester bond in an oligonucleotide is converted to a peptide-nucleic acid bond, an oligonucleotide derivative wherein the uracil in an oligonucleotide is substituted by C-5 propynyluracil, an oligonucleotide derivative wherein the uracil in an oligonucleotide is substituted by C 5 thiazolyluracil, an oligonucleotide derivative wherein the cytosine in an oligonucleotide is substituted by C-5 propynylcytosine, an oligonucleotide derivative wherein the cytosine in an oligonucleotide is substituted by phenoxazine-modified cytosine, an oligonucleotide derivative wherein the ribose in DNA is substituted by 2'-O-propylribose, and an oligonucleotide derivative wherein the ribose in an oligonucleotide is substituted by 2'-methoxyethoxyribose.
- 25 16. A method for detecting an mRNA encoding the polypeptide according to any of Claims 1 to 3, which comprises using the oligonucleotide according to any of Claims 13 to 15.
- 30 17. A method for inhibiting the expression of the polypeptide according to any of Claims 1 to 3, which comprises using the oligonucleotide according to any of Claims 13 to 15.
- 35 18. An antibody recognizing the polypeptide according to any of Claims 1 to 3.
19. A method for immunological detection of the polypeptide according to any of Claims 1 to 3, which comprises using the antibody according to Claim 18.
- 40 20. A method for immunohistochemical staining of the polypeptide according to any of Claims 1 to 3, which comprises using the antibody according to Claim 18.
21. An immunohistochemical staining agent comprising the antibody according to Claim 18.
- 45 22. A method for screening for a compound varying the phospholipase A₂ activity of the polypeptide according to any of Claims 1 to 3, which comprises contacting said polypeptide with a test sample, and measuring the phospholipase A₂ activity of said polypeptide.
- 50 23. A method for screening for a compound varying the expression level of the polypeptide according to any of Claims 1 to 3, which comprises contacting cells expressing said polypeptide with a test sample, and detecting the expression level of said polypeptide.
- 55 24. The method according to Claim 23, wherein said detection of the expression level of said polypeptide is detection of an mRNA encoding the polypeptide according to any of Claims 1 to 3 using the method according to Claim 16.
25. The method according to Claim 23, wherein said detection of the expression level of said polypeptide is detection of the polypeptide using the method according to Claim 19.
26. The method according to Claim 22, wherein said variation of the phospholipase A₂ activity of the polypeptide

according to any of Claims 1 to 3 is an increase in the phospholipase A₂ activity of said polypeptide.

27. The method according to Claim 22, wherein said variation of the phospholipase A₂ activity of the polypeptide according to any of Claims 1 to 3 is a decrease in the phospholipase A₂ activity of said polypeptide.

28. The method according to any of Claims 23 to 25, wherein said variation of the expression of the polypeptide according to any of Claims 1 to 3 is an increase in the expression level of said polypeptide.

29. The method according to any of Claims 23 to 25, wherein said variation of the expression of the polypeptide according to any of Claims 1 to 3 is a decrease in the expression level of said polypeptide.

30. A compound which is obtainable by the method according to any of Claims 22 to 29.

31. A promoter DNA regulating the transcription of a DNA encoding the polypeptide according to any of Claims 1 to 3.

32. A method for screening for a compound varying the efficiency of transcription of a DNA encoding the polypeptide according to any of Claims 1 to 3, which comprises contacting a transformant carrying a plasmid containing the promoter DNA according to Claim 31 and a reporter gene ligated downstream of said promoter DNA with a test sample, and measuring the content of the translation product of said reporter gene.

33. The method according to Claim 32, wherein the reporter gene is a gene selected from the group consisting of a chloramphenicol acetyltransferase gene, a β -galactosidase gene, a luciferase gene, a β -glucuronidase gene, an aequorin gene and a green fluorescent protein gene.

34. The method according to Claim 32 or 33, wherein said variation of the efficiency of transcription of a DNA encoding the polypeptide according to any of Claims 1 to 3 is an increase in the efficiency of transcription of said DNA.

35. The method according to Claim 32 or 33, wherein said variation of the efficiency of transcription of a DNA encoding the polypeptide according to any of Claims 1 to 3 is a decrease in the efficiency of transcription of said DNA.

36. A compound which is obtainable by the method according to Claims 32 to 35.

37. A polypeptide consisting of an amino acid sequence wherein a part or the whole of the amino acid sequence of the active domain is deleted in the amino acid sequence of the polypeptide according to any of Claims 1 to 3.

38. A polypeptide consisting of the amino acid sequence shown in SEQ ID NO: 3.

39. A polypeptide consisting of an amino acid sequence wherein one or more amino acid residues are deleted, substituted or added in the amino acid sequence shown in SEQ ID NO: 3 and having the activity of inhibiting phospholipase A₂ activity.

40. A polypeptide consisting of an amino acid sequence which has 60% or more homology to the amino acid sequence shown in SEQ ID NO: 3 and having the activity of inhibiting phospholipase A₂ activity.

41. A DNA encoding the polypeptide according to any of Claims 37 to 40.

42. A DNA having the nucleotide sequence shown in SEQ ID NO: 4.

43. A DNA which hybridizes to a DNA consisting of a nucleotide sequence complementary to the nucleotide sequence shown in SEQ ID NO: 4 under stringent conditions and which encodes a polypeptide having the activity of inhibiting phospholipase A₂ activity.

44. A recombinant vector comprising the DNA according to any of Claims 41 to 43.

45. A transformant carrying the recombinant vector according to Claim 44.

46. The transformant according to Claim 45, wherein the transformant is selected from the group consisting of a microorganism, an animal cell, a plant cell and an insect cell.

47. A process for producing a polypeptide having the activity of inhibiting phospholipase A₂ activity, which comprises culturing the transformant according to Claim 45 or 46 in a medium, allowing the polypeptide having the activity of inhibiting phospholipase A₂ activity to form and accumulate in the culture, and recovering the polypeptide from the culture.

48. A pharmaceutical for the diagnosis, prevention or treatment of a disease in which the polypeptide according to any of Claims 1 to 3 is concerned, which comprises, as an active ingredient, a compound varying the phospholipase A₂ activity of said polypeptide.

49. A pharmaceutical for the diagnosis, prevention or treatment of a disease in which the polypeptide according to any of Claims 1 to 3 is concerned, which comprises said polypeptide as an active ingredient.

50. A pharmaceutical for the diagnosis, prevention or treatment of a disease in which the polypeptide according to any of Claims 1 to 3 is concerned, which comprises, as an active ingredient, the DNA according to any of Claims 4 to 6.

51. A pharmaceutical for the diagnosis, prevention or treatment of a disease in which the polypeptide according to any of Claims 1 to 3 is concerned, which comprises, as an active ingredient, the polypeptide according to any of Claims 37 to 40.

52. A pharmaceutical for the diagnosis, prevention or treatment of a disease in which the polypeptide according to any of Claims 1 to 3 is concerned, which comprises, as an active ingredient, the DNA according to any of Claims 41 to 43.

53. A pharmaceutical for the diagnosis, prevention or treatment of a disease in which the polypeptide according to any of Claims 1 to 3 is concerned, which comprises, as an active ingredient, the oligonucleotide according to any of Claims 13 to 15.

54. A pharmaceutical for the diagnosis, prevention or treatment of a disease in which the polypeptide according to any of Claims 1 to 3 is concerned, which comprises, as an active ingredient, the antibody according to Claim 18.

55. A pharmaceutical for the diagnosis, prevention or treatment of a disease in which the polypeptide according to any of Claims 1 to 3 is concerned, which comprises, as an active ingredient, the compound according to Claim 30 or 36.

56. The pharmaceutical according to any of Claims 48 to 55, wherein said disease in which said polypeptide is concerned is asthma, ischemic diseases, arthritis, rheumatism, sepsis, dermatitis, arteriosclerosis, pain, Parkinson disease, Alzheimer disease, malignant tumor, nephritis, diabetes or ischemic reperfusion injury.

57. A pharmaceutical for the diagnosis, prevention or treatment of diabetes, which comprises, as an active ingredient, a compound obtainable by the method according to Claim 28 or 34.

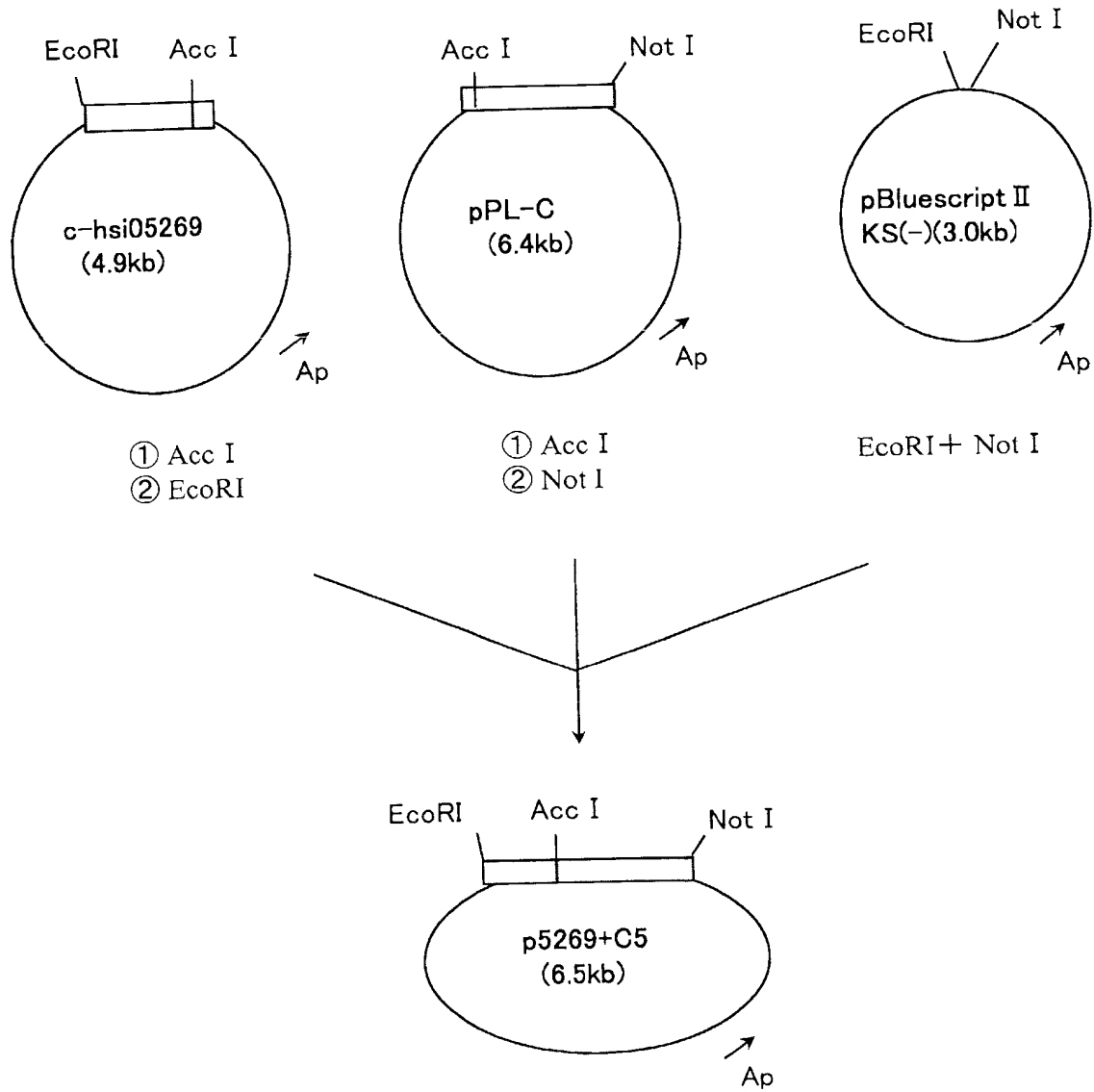


Fig. 1

75

477' AKLSDQQEAVRQGNPYPIYTSVNVRTNLSGEDFAEWCEFTPYEVGFPPKYGAYVPTFLFG
 ***. . * * * * * * * * * * * * * * * * * * * *
 310" TTLSLKEKVNTAQCPPLFLTCLHVKPPDVSELMFADWVEFSPYEIGMAKYGTFMAPDLFG

 537' SELFMGRLLQLQPEPRICYLQGMWGSAFATSLDEIF---LKTAGSGL-SFLEWYRGSVN
 ***** * . . * * * * * * * * * * * * * * *
 370" SKFFMGTVVKKYEENPLHFLMGVWGSFAFSLFNRLVGVSGSQSRGSTMEEELENITTKHI

 592' ITDDCQKPKQLHNPSRLRTRLLTPQGPF-----SQAVLDDIFTSRFT-SAQS
 ***. * * * *
 430" VNSDSSDDESHEPKGTENEDAGSDYQSDNQASWIHRMINALVSDSALFNTRREGRAKV

 636' FNFTRGCLCHKDY--VAGREFVA---WKDTH-----PDAFPNQLTPM---RDCLYLVD
 ** ** * * * * * * * * * * * * * * * * * * * *
 490" HNFMLGLNLNTSYPLSPLSDFATQDSFDDDELDAAVADPDEFERIYEPLDVKSKKIHVVD

 681' GGFAINSPFPLALLPQRAVDLILSFDYSL-----EAPFEVLKMTKEYCLDRGIPFPSIEV
 * * * * * * * * * * * * * * * * * * * *
 550" SGLTFNLPYPLILRPQRGVDLIIISFDFSARPSDSSPPFKELLLAEKWAKMNKLPFPKIDP

 736' GPEDVEEARECYLF--AKAEDPRSPIVLHFPLVNRTRTHLAPGVERQTAEKAFGDF-
 * * * * * * * * * * * * * * * * * *
 610" YVFDREGLKECYVFKPKNPDMEKDCPTIIHFVLANINFRKYKAPGVPRETEEEKEIADFD

 792' VINRPDTPYGMNFTYPEQDFYRLVALSRYNVLNNVETLKCALQLALD-RHQARERAGA
 ... * * * * * * * * * * * * * * * * * * * *
 670" IFDDPESPSTFNFPNQAFKRLHDLMHFNTLNNIDVIKEAMVESIEYRRQNPSRCSVS

76
 3

1' MLWALWPRWLADKMLPLLGAULLQKREKRGPLWRHWRRETPY

181" HYENLYCVVSGEKHFLFHPPSDRPFIPYELYTPATYQLTEEGTFKVVDDEAMEKAEVSR

44' YDLQVKVLRATNIRGTDLLSKADCYVQLWLPATASPAQTRIVANCSDPENNETFHYQIH
* * * * * * * * * * * * * * * * * * * *

241" CLLTVRVLQAHRLPSKDLVTPSDCYVTLWLPTACSHRLQTRTVKNSSSPVWNQSFHFRIH

104' GAVKNVLELTLYDKDILGSDQLSL-LLFDLRLSLKCGQPHKHTFPLNHQDSQELQVEFVLE
* * * * * * * * * * * * * * * * * * * *

301" RQLKNVMELKVFDQDLVTGDDPVLVDFDAGTLRAGEFRRESFSLSPQGEGRLEVEFRLQ

163' KSQVPASEVITNGVLVAH--PCIRIQTLRGDGTAPREEYSGQLQLAVPGAYEKPQLLP
. * * * * * * * * * * * * * * *

361" SLADRGEWLVSNGVLVARELSCLHVQ--L--EETGD-QKSSEHRVQLVVPGSCEGPQ--
. * * * * * * * * * * * * * * *

221' LQPPTEPGLPPTTFHFVNPVLSRLHVELMELLAQVQSGPSTELEAQTSLGEGGILLSS
. * * * * * * * * * * * * * * *

413" -EASVGTG--TFRFHCPCWQEQLSIRLQD-----APEEQLKAPLSALPSGQVVRLV
. * * * * * * * * * * * * * * *

281' LPLGQEEQCSVALGEGQEVALSMMKVEMSSGDLRLGLFDLSDGEQEFLLDRRKQVVSALQ
. * * * * * * * * * * * * * * *

462" FPTSQ-EP-----LMRVELKKEAGLRELAVRLGFGPCAEQAFLSRRKQVVAALR
. * * * * * * * * * * * * * * *

341' QVLGLSEALDSGQVPVAVLGSCTRAMSSLYGSLAGLQELGLDVTYLSGVSGSTWC
* * * * * * * * * * * * * * * * * * * *

512" QALQLDGDLDQDEIPVVAIMATGGGIRAMTSLYQLAGLQELGLDCVSYITGASGSTWA

Fig. 4

401' ISTLYRDPAWSQVALQGPIERAQVHVCSKMGALSTERLQYTYTOELGVRERSGHSVSLID
 ...** ** ** ** *** ...* ...* ...* ...* ...* ...* ...* ...* ...*
 572" LANLYEDPEWSQKDLAGPTTELLKTQVTKNKLGVLAQPSQLQRYRQELAERARLGYPSCFN

 461' LWGLLVEYLLYQEEENPAKLSDDQEAQRQGNPYPIYTSVNVRT-NLSGEDFAEWCEFTPY
 .* * **.* . ***.*...***** **.*... **.*...*****.*
 632" LWALINEALLHDEPHDKLSDQREALSHGQNPLPIYCALNTKGQSLTTFFEGWCEFFSPY

 520' EVGFPKYGAYVPTTELFGSELFMGRLLQLQPEPRICYLQGMWGSFAFATSLDEIFLKTAGSG
 *****.*...*****.*... *****.*... *****.*... *****.*
 692" EVGFPKYGAFIPSELFGSEFFMGQLMKRLPESRICFLEGIWSNLYAANLQDSLYWASEPS

 580' LSFLEWYRGSVNITDDCQKQPLHNPSRLRTRLLTPQGFPSQAVLDIFTSRFTSAQSFNFT
 . * *. *... * * * * .. *... *... *... *... *... *
 752" QFWRWVRNQANLKE-QVPLLKIEP-----PSTAGRIAEFFTDLLTWRPLAQATHNFL

 640' RGLCLHKDYVAGREFVAVKDTHPDAFPNQLTPMRDCLYLDGGFAINSP-FPLALLPQRA
 * . * . * . * . * . * . * . * . * . * . * . * . * . * . * . * .
 806" RGLHFHKDYFQHPHFSTWKATTLDGLPNQLTPSEPHLCCLLDVGYLINTSCLPL-LQPTRD

 699' VDLILSFDYSLEAPFEVLKMTKEYCLDRGIPFPFPIEVGPEDEEARECYLFAKAEDPRSP
 *****.*... * . . . * * * * * .
 865" VDLILSLDYNLHGAFQQLQLLGRFCQEQGIPFPPISPPEEQQLQPRECHTFSDPTCPGAP

 759' IVLHFPLVNRTRTHLAPGVERQTAEKAFGDFVINRPDTPYGMNFTYEPQDFYRLVAL
 *****.*... ***** * . . . * * * * .
 925" AVLHFPLVSDSFREYSAPGV-RRTPEEAAAGEVNLSDDSPYHYTKVTYSQEDVDKLLHL

 819' SRYNVLNNVETLKCALQLALDRHQARERAGA
 ..*** ** * * **.*...*
 984" THYNVCNNQEQLLEALRQAVQRRRRQRRPH

$\frac{1}{100}$ 5

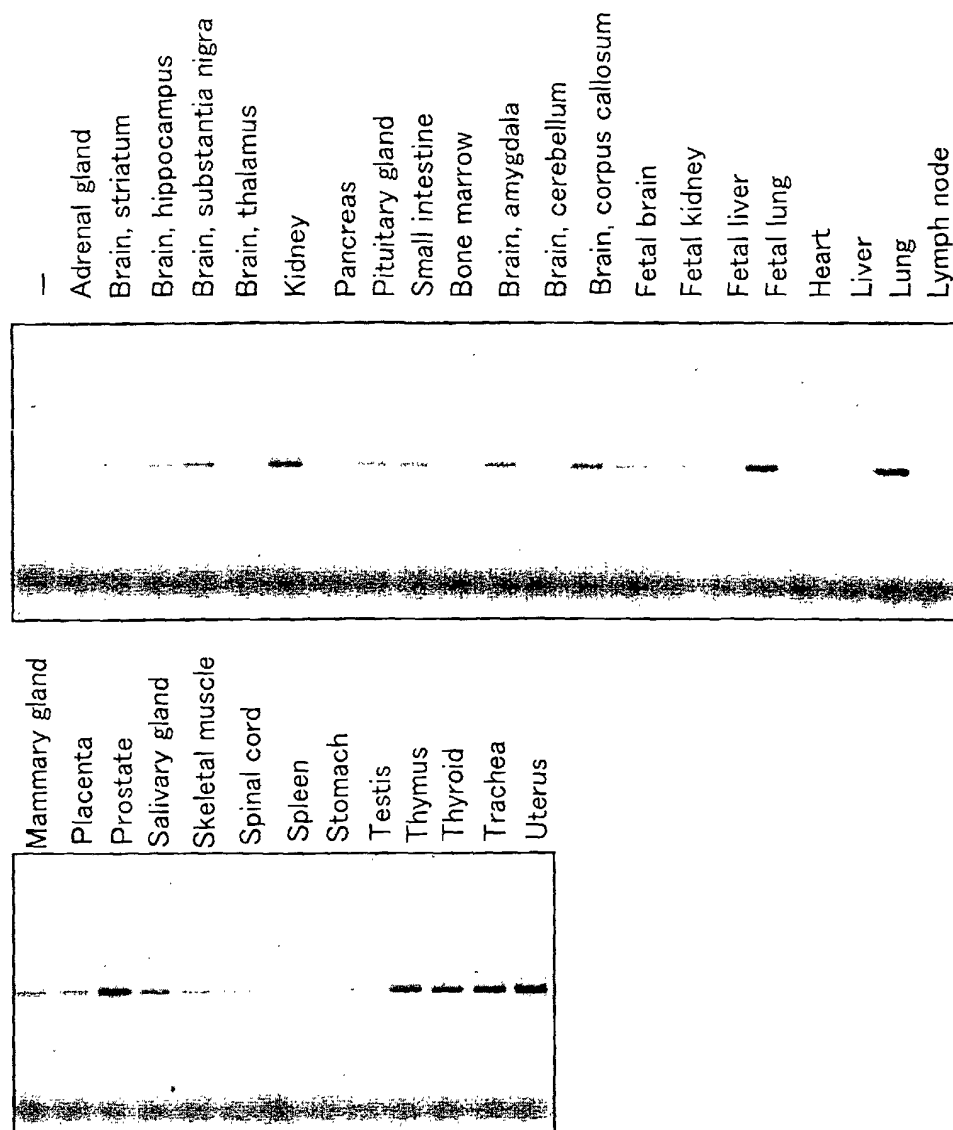


Fig. 6

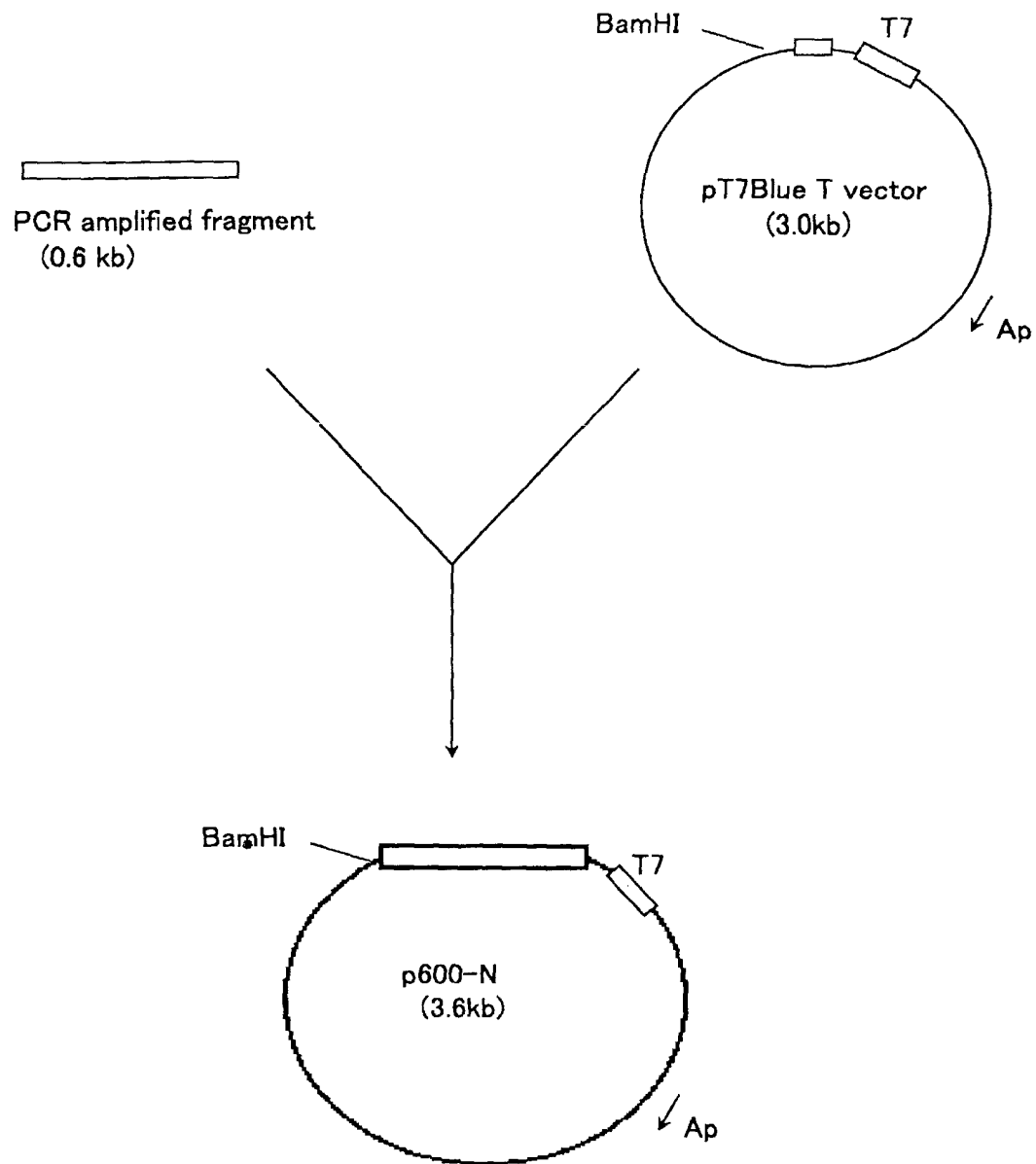


Fig. 7

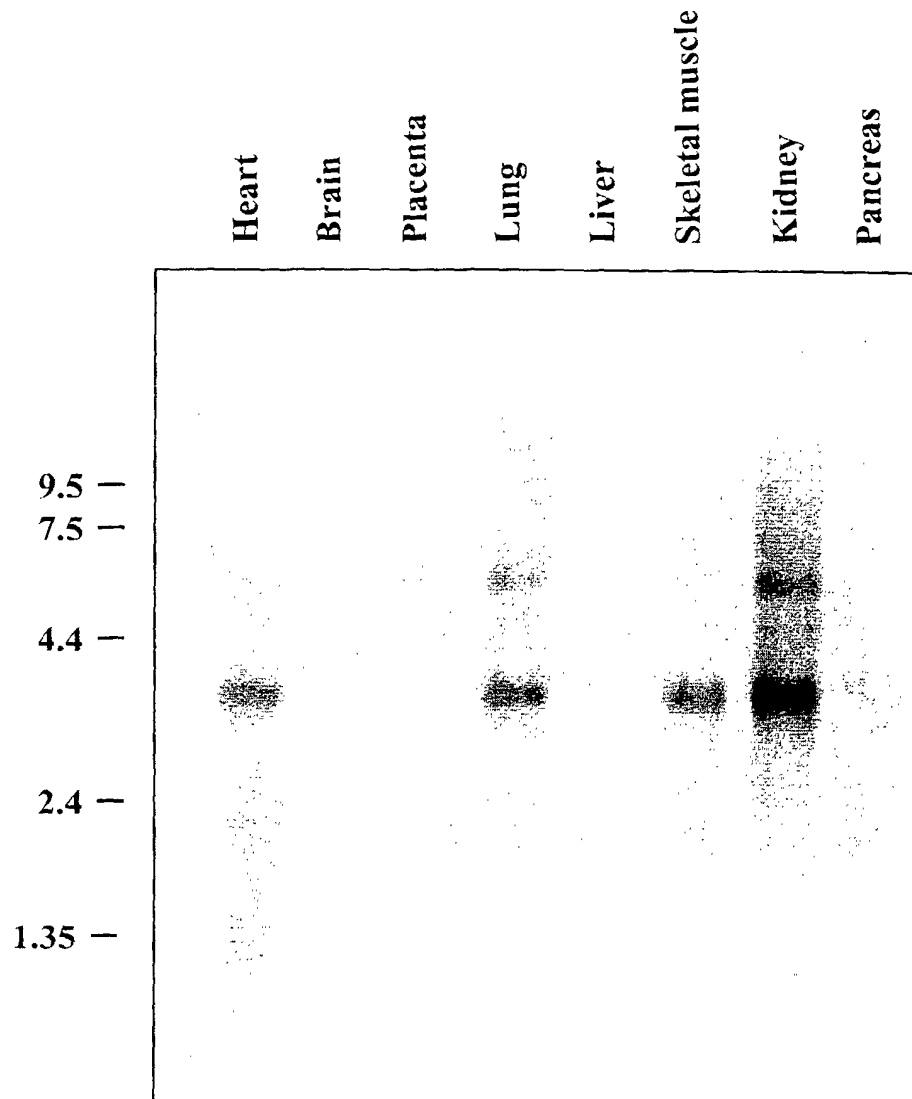


Fig. 8

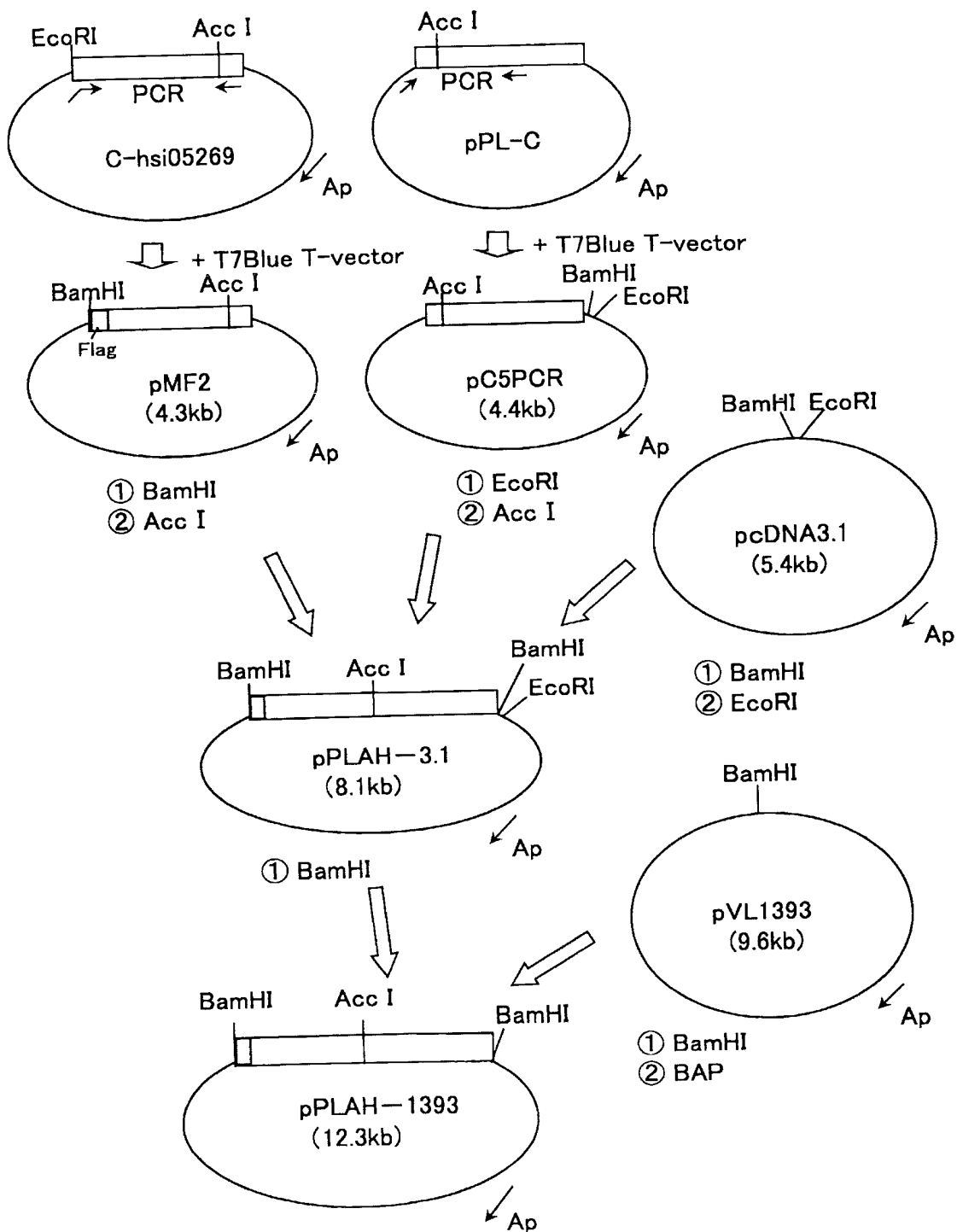


Fig. 9

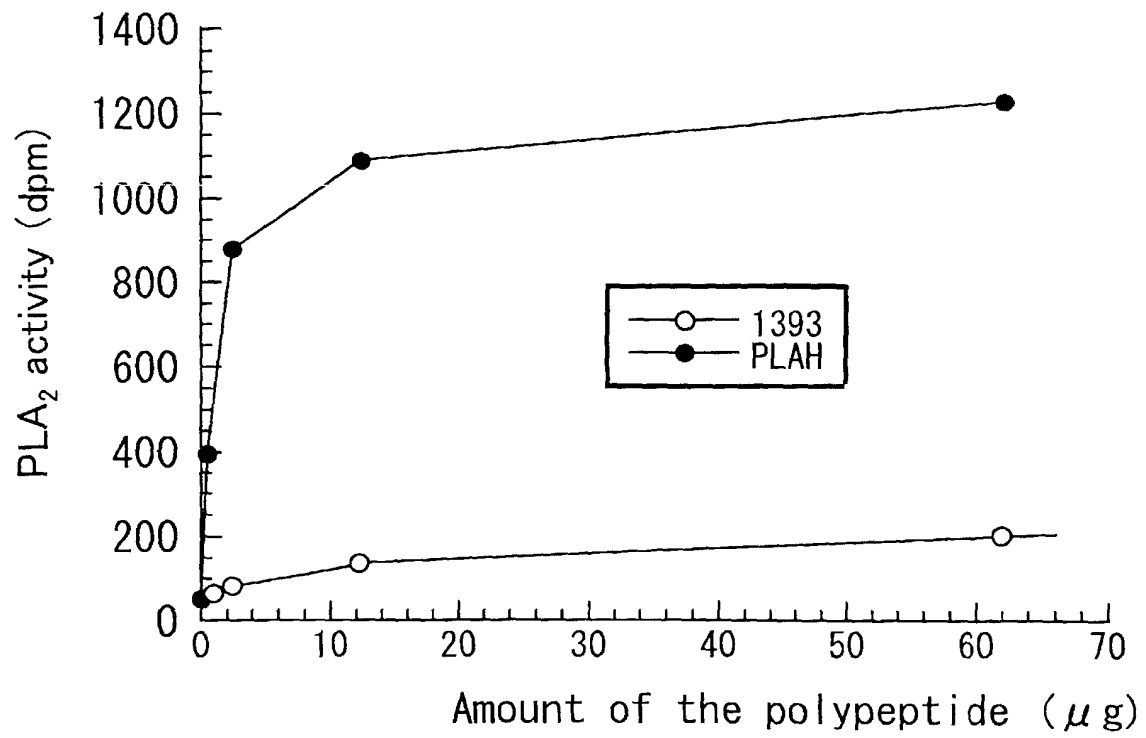


Fig. 10

```

1' MLWALWPRWL ADKMLPLLGA VLLQKREKRG PLWRHWRRET YPYDDLQVKV LRATNIRGTD
* * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
1" MPWTLQPKWL AGKGLPLLGA ILLRKTEKSE PQWKH-RRET HPYDDLQVKV LRARNIQHTD

61' LLSKADCYVQ LWLPTASVSP AQTRIVANCS DPEWNETFHY QIHGAVKNVL ELTYDKDIL
* * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
60" KLSKADCYVR LWLPTASVSP SQTRTVVNSS DPEWNETFHY QIHGAVKNVL ELALYDEDEVL

121' GSDQLSLLLF DLRLSKCGQP HKHTFPLNHQ DSQELQVEFV LEKSQVPASE VITNGVLVAH
. * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
120" DSDNVFSILF DMSTLQLGQP CTKNFT-RQQ DPKELEVEFT LEKSQTPASE VVTNGVLVAH

181' PCLRIQGTLR GDGTAPREEY GSGQLQLAVP GAYEKPQLLP LQPPTEPGLP PTFTFHVNPV
* * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
179" PCLRIQGTVT GDKTASLGEL GSRQIQLAVP GAYEKPQ--P LQPTSEPLP VNFTFHMNPV

241' LSSRLHVELM ELLAAVQSGP STELEAQTSK LGEGGILLSS LPLGQEEQCS VALGEGQEVA
* * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
237" LSPKLHIKLQ EQLQVFHSGP SDELEAQTSK MDKASILLSS LPLNEELTKL VDLEEGQQVT

```

Fig. 11

```

301' LSMKVEM-SS GDLDLRLGFD LSDGEQEFLD RRKQVVS KAL QQVLGLSEAL DSGQVPVVAV
*.*.*.*.* ** ***** *.*.*.*.* **.*.*.*.* **.*.*.*.* **.*.*.*.*
297" LRMKADMSSS GDLDLRLGFD LCDGEQEFLD KRKQVASKAL QRVMLGLSEAL HCDQVPVVAV

360' LSGGGGTRAM SSLYGSLAGL QELGLLDTVT YLSGVSGSTW CISTLYRDP A WSQVALQGPI
*****.*.*.*.* ** *****.*.*.*.* **.*.*.*.* **.*.*.*.*
357" LSGGGGTRAM TSLYGSLAGL QELGLLDAVT YLSGVSGSSW CISTLYRDP S WSQKALQGPI

420' ERAQVHVCSS KMGALSTERL QYYTQELGVR ERSGHVS VLI DLWGLLVEYL LYQEE NPAKL
.*.*.*.*.* **.*.*.*.* **.*.*.*.* **.*.*.*.* **.*.*.*.*
417" KYASERVCSS KIGMLSPKQF EYSREKRAW ESRGHMSMSFT DLWGLIIEYF LNQEE NPAKL

480' SDQQEAVRQG QNPYPIYTSV NVRTNLSGED FAEWCEFTPY EVGF PKYGAY VPTELF GSEL
*****.*.*.*.* **.*.*.*.* *****.*.*.*.* *****.*.*.*.*
477" SDQQETVSQG QNPYPIYASI NVHKNISGDY FAEWCEFTPY EVGF PKYGVY VPTELF GSEF

```

Fig. 12

```

540' FMGRLLQLQP EPRICYLQGM WGSAFATSLD EIFLKTAGSG LSFLEWYRGS VNITDDCQKP
***** * ***** * ***** * ***** * ***** *
537" FMGRLLHFWP EPRICYLQGM WGSAAFAASLY EIFLKLGGLS LSFLDWHRGS VSVTDDWPKL

600' QLHNPSRLRT RLLTPQGPFS QAVLDIFTSR FTSAQSFNFT RGLCLHKDYV AGRF---V
. . . . . * * * * * * * * * * * * * * * * * *
597" RKQDPTRLPT RLFTPMSFS QAVLDIFTSR ITCAQTFNFT RGLCMYKDYT ARKDFVVSSED

656' AW---KDTHP DAFPNQLTPM RDCLYLVDGG FAINSPFPLA LLPQRAVDLI LSFYDYSLEAP
* * . . . * * * * * * * * * * * * * * * * * *
657" AWHSHNYGYP DACPNQLTPM KDFLSLVDGG FAINSPFPLV LQPQRAVDLI VSFYDYSLEGP

713' FEVLKMTKY CLDRGIPFPS IEVGPEDVEE ARECYLFAKA EDPRSPIVLH FPLVNRFTFT
***** * * * * * * * * * * * * * * * * * * * * *
717" FEVLQVTEKY CRDRGIPFPR IEVDPKDSER PRECYLFTEA EDPCSPIVLH FPLVNRFTFT

773' HLAPGVERQT AEEKAFGDFV INRPDTPYGM MNFTYEPQDF YRLVALSRYN VLNNVETLKC
***** * * * * * * * * * * * * * * * * * * * *
777" HLAPGVERQT AEEKAFGDFI INGPDTAYGM MDFTYEPKEF DRLVTLSRYN VLNNKETIRH

833' ALQLALD-RH QARERAGA
***** * * * * *
837" ALQLALDRRR QAGGRVGG

```

Fig. 13

```

1' YLQGMWGSF AASLYEIFLK MRGPRLGFLD WHRGTVSVTD DWPKLKQDDP TRLPTRLFTS
***** * . * . * . * . * . * . * . * . * . * . * . * . * . * . * .
1" YLQGMWGSF ATSLDEIFLK TAGSGLSFLE WYRGSVNITD DCQKQQLHNP SRLRTRLTTP

61' KSFFSKAVLD IFTSRFTCAQ TNFTRGLCL YKDYTARKDF VVSEDAWHSN NYKHLDACPN
.. * . * . * . * . * . * . * . * . * . * . * . * . * . * . * .
61" QGPFSQAVLD IFTSRFTSAQ SNFTRGLCL HKDYVAGREF VAWKDT-HPD AF-----PN

121' QLTPMKDFLS LVDGGFAINS PFPLILQPQR AVDLIVSFDY SLEAPFEVLQ VTEKYCRDRG
***** * . * . * . * . * . * . * . * . * . * . * . * . * . * . * .
114" QLTPMRDCLY LVDGGFAINS PFPLALLPQR AVDLILSFDY SLEAPFEVLK MTEKYCLDRG

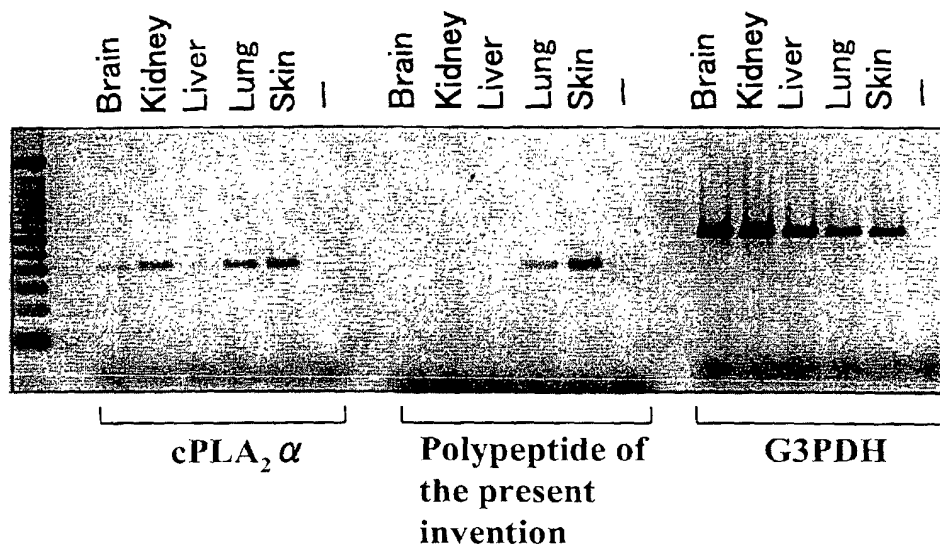
181' IPFPRIEVDP KDSKDPRECY LFTEAEDPCS PIVLHFPLVN RTRKHLAPG VERQTAEKA
***** * . * . * . * . * . * . * . * . * . * . * . * . * . * . * .
174" IPFPSIEVGP EDVEEARECY LFKAEDPRS PIVLHFPLVN RTRTHLAPG VERQTAEKA

241' FGDFIINGPD TAYGMNFTY E
***** * . * . * . * . * . * . * . * . * . * . * . * . * . * . * .
234" FGDFVINRPD TPYGMNFTY E

```

Fig. 14

Mouse



Rat

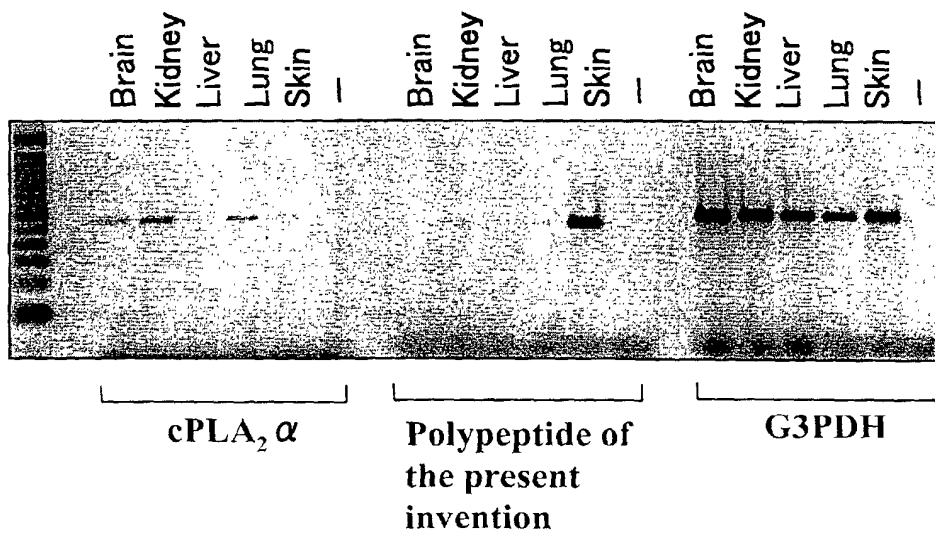


Fig.15

1: MLWALW - PRWLADKMLPLLGAULLQKREKRGLWRHRRRETYPYDYLQVKVLRATNIRGT 59
1: MPWT - LQPKWLAGKGLPLLGAULLRKTSEKSEPOWKHR - RETHPYDYLQVKVLRARNIQHT 58
1: MPWT - LQPKWLAGKGLPLLGAULLRKTSEKSEPOWKHR - RETHPYDYLQVKVLRARNIQHT 58
* * * * *
60: DLLSKADCYVQLWLPTASPSPAQTRIVANCSDPEWNETFHYQIHGAVKNVLELTLYDKDI 119
59: DKLSKADCYVRLWLPTASVSPSQTRIVNSSDPENNETFHYQIHGAVKNVLELALYDEDV 118
59: DKLSKADCYVRLWLPTASVSPSQTRIVNSSDPENNETFHYQIHGAVKNVLELALYDEDV 118
* * * * *
1120: LGSDDL - SLLFDLRLSLKCGOPH - KHTFPLNHQDSQELQVEFVLEKSQVPASEVITNGVL 177
1119: LDSDNVFSIL - FDMSTLQLGQPCTKN - FTRQ - QDPKELEVEFTLEKSQTPASEVITNGVL 175
1119: LDSDNVFSIL - FDTSTLQLGQPCTKN - FTRQ - QDPKELEVEFTLEKSQTPASEVITNGVL 175
* * * * *
11178: VAHPCLRIOGTLRGDGTAPREEYVSGQLQLAVPGAYEKPQLLPQPTEPGLPPTFTFHV 237
11176: VAHPCLRIOGTLRGDGTASLGELSRQIQLAVPGAYEKP - QPLQPTSEPLPVNFTFHM 233
11176: VAHPCLRIOGTLRGDGTASLGELSRQIQLAVPGAYEKP - QPLQPTSEPLPVNFTFHV 233
* * * * *
2238: NPVLSSRLHVELMELLAUVQSGPSTELFAQTSKLGEGGILLSSILPLGQEEQCSV - ALGEG 296
2234: NPVLSPKLHIKLEQLQVFHSGPSDELEAQTSMKDKASILSSILPLNE - ELTKLVDLLEEG 292
2234: NPVLSPKLHIKLEQLQVFHSGPSDELEAQTSMKDKASILSSILPLNE - ELTKLVDLLEEG 292
* * * * *
2297: QEVALSMKVEMSSG - DLDLRLGFDLSDGGEQEFLDRLKQVVKALQQVGLGLSEALDSGQVP 355
2293: QQVTLRMKADMSSGDLRLGFDLSDGGEQEFLDRLKQVVKALQVRMGLSEALHCDQVP 352
2293: QQVSLRMKADMSSG - DLDLRLGFDLSDGGEQEFLDRLKQVVKALQVRMGLSEALHCDQVP 351
* * * * *
3356: VVAVLGSGGGTRAMSSLYGSLAGLQELGLLDVTYLSGVSGSTWCISTLYRDPAWSQVAL 415
3353: VVAVLGSGGGTRAMTSLYGLAGLQELGLLDVAVTYLSGVSGSSWCISTLYRDPAWSQVAL 412
3352: VVAVLGSGGGTRAMTSLYGLAGLQELGLLDVAVTYLSGVSGSSWCISTLYRDPAWSQVAL 411
* * * * *
416: QGPIERAQV - HVCSSKMGMALSTERLOYYTQELGVRE - RSGHVSLSIDLWGLLVEYLLYQE 473
413: QGPIKYA - SERVCSSKIGMLSPKQFEYYSREKAWESR - GHMSFTDLWGLIIEYFLNQE 470
412: QGPIKYA - SERVCSSKIGMLSPKQFEYYSREKAWESR - GHMSFTDLWGLIIEYFLNQE 469
* * * * *

```

474:ENPAKLSDDQEA VRQGNPYPIYTSVNVRTNLSGED-FAEWCEFTPYEVGFPKYGAYVPT 532
471:ENPAKLSDDQETVSQGNPYPIYASINVHKNISG-DYFAEWCEFTPYEVGFPKYGVVPT 529
470:ENPAKLSDDQETVSQGNPYPIYASINVHKNISGDD-FAEWCEFTPYEVGFPKYGAYVPT 528
*****
533:ELFGSELFMGRLLQ-LQPEPRICYLQGMWGSFAFATS-LDEIFLKTAGSGLSFLEWYRGSV 590
530:ELFGSEFFMGRLLHFW-PEPRICYLQGMWGSFAFAASLY-EIFLKLGLSLSFLDWHRGSV 587
529:ELFGSEFFMGRLLHFW-PEPRICYLQGMWGSFAFAASLY-EIFLKLGLSLSFLDWHRGSV 586
*****
591:NITDDCQKQPLHN--PSRLRTRLTPQGPFSQAVLDIFTSRFTSAQSFNTRGLCLHKDY 648
588:SVTD--DWPKLKQDPTRLPTLTPMSSFSQAVLDIFTSRITCAQTFNTRGLCMYKDY 645
587:SVTD--DWPKLKQDPTRLPTLTPMSSFSQAVLDIFTSRITCAQTFNTRGLCMYKDY 644
*****
649:VAGR-EFV---AWKDTHPDA--F---PNQLTPMRDCLY--LVDGGFAINSPPFLALLP 695
646:TA-RKDFVUSED AWHs-H--NYGYPDACPNQLTPMK-D-FLSLVDGGFAINSPPFLVLQP 699
645:TA-RKDFVUSED AWHs-H--NYGYPDACPNQLTPMK-D-FLSLVDGGFAINSPPFLVLQP 698
*****
696:QRAVDLILSFDYSLEAPFEVLKMTKEYCLDRGIPFPSPSIEVGPEDVEEARECYLFAKAEDP 755
700:QRAVDLIVSFDYSLEGPFEVLQVTEKYCRDRGIPFPRIEVDPKDSEDPRECYLFTAEADP 759
699:QRAVDLIVSFDYSLEGPFEVLQVTEKYCRDRGIPFPRIEVDPKDSEDPRECYLFAEADP 758
*****
756:RSPIVLHFPLVNRTRFTHLAPGVERQTAEKAFGDFVINRPDTPYGMNFTYEPQDFYRL 815
760:CSPIVLHFPLVNRTRFTHLAPGVERQTAEKAFGDFIINGPDTAYGMMDFTYEPKEFDRL 819
759:CSPIVLHFPLVNRTRFTHLAPGVERQTAEKAFGDFIINGPDTAYGMMDFTYEPKEFDRL 818
*****
816:VALSRYNVLNNVETLKCALQLALDRHQ-ARERAGA 849
820:VTLSRYNVLNNKETIRHALQLALDRRRQAGGRVGG 854
819:VTLSRYNVLNNKETIRHALQLALDRRRQAGGRVGG 853
*****

```

Fig. 17

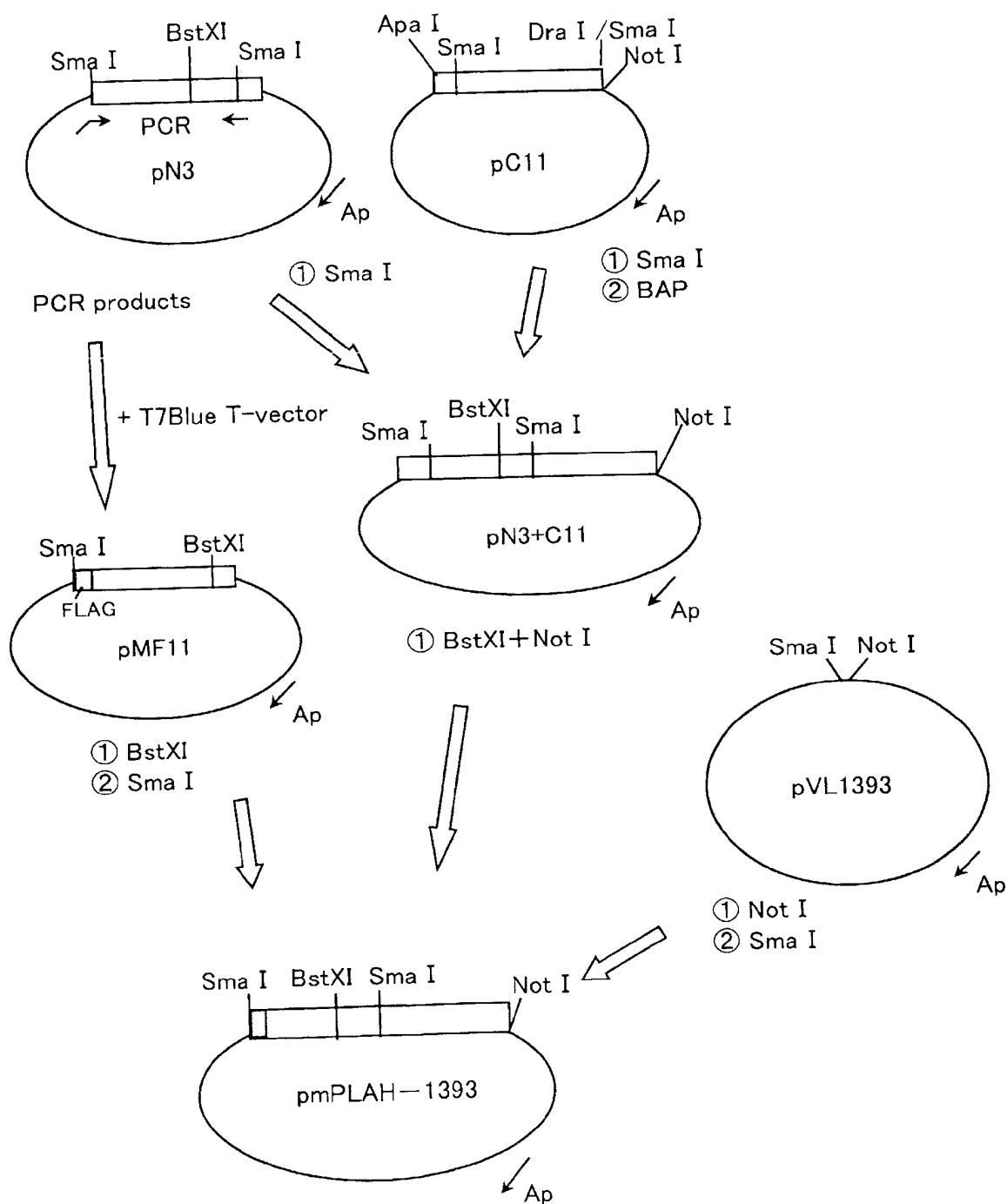


Fig. 18

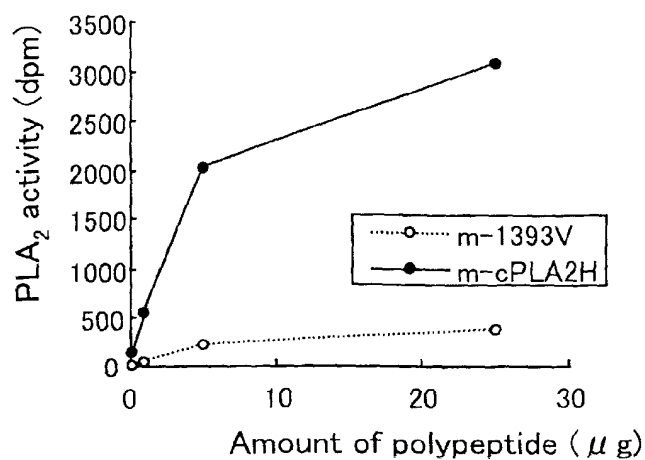


Fig. 19

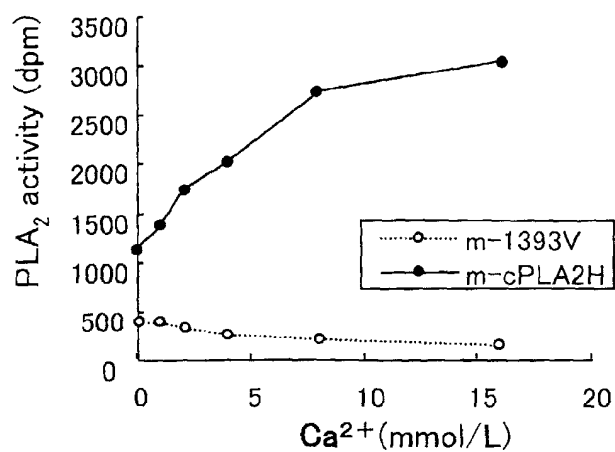


Fig. 20

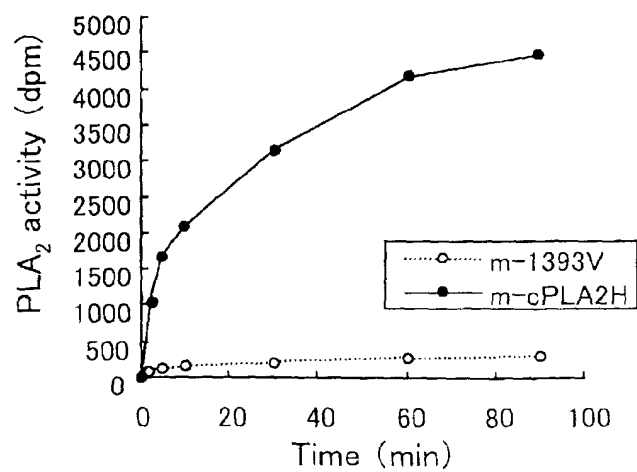


Fig. 21

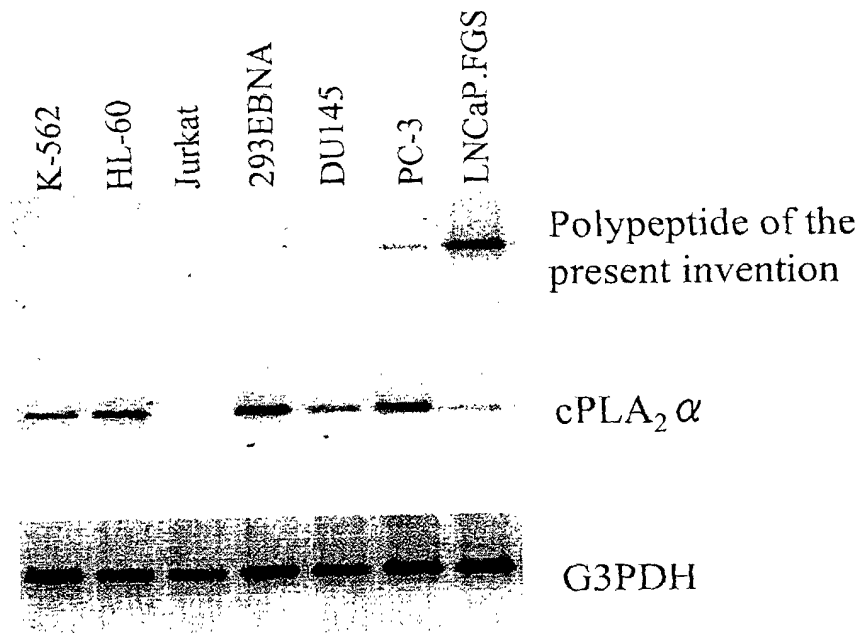


Fig. 22

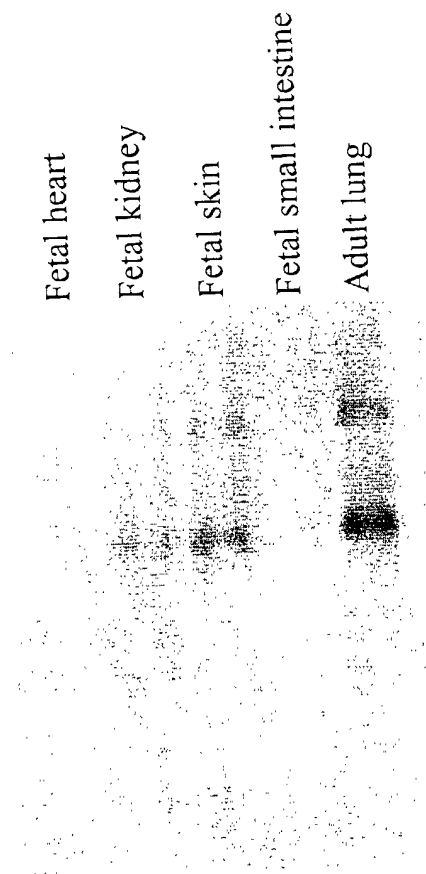


Fig. 23

INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP01/08138

A. CLASSIFICATION OF SUBJECT MATTER Int.Cl ⁷ C12N15/55, C12N9/16, C12N5/10, C12N1/21, C12Q1/68, C07K16/40, G01N33/573, G01N33/50, G01N33/15, A61K38/46, A61K31/711, A61K39/395, A61P11/06, A61P9/10, A61P19/02, A61P39/00, A61P7/00, A61P17/00, A61P25/16, A61P5/38, A61P25/28, A61P35/00, A61P13/12		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) Int.Cl ⁷ C12N15/55, C12N9/16, C12N5/10, C12N1/21, C12Q1/68, C07K16/40, G01N33/573, G01N33/50, G01N33/15, A61K38/46, A61K31/711, A61K39/395, A61P11/06, A61P9/10, A61P19/02, A61P39/00, A61P7/00, A61P17/00, A61P25/16, A61P5/38, A61P25/28, A61P35/00, A61P13/12		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) JICST FILE (JOIS), WPI (DIALOG), BIOSIS (DIALOG), MEDLINE (STN), EMBL/DBBJ/Genbank/PIR/Swissprot/Geneseq		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 6025178 A (Lilly & Co. Eli), 15 February, 2000 (15.02.00), (Family: none)	1-35, 37-57
A	WO 00/24911 A2 (Incyte Pharm. Inc.), 04 May, 2000 (04.06.00), & AU 200014516 A & EP 1131445 A1	1-35, 37-57
A	WO 00/47763 A1 (Genetics Inst. Inc.), 17 August, 2000 (17.08.00), & AU 200029937 A	1-35, 37-57
<input type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
* "A" "E" "L" "O" "P"	Special categories of cited documents: document defining the general state of the art which is not considered to be of particular relevance earlier document but published on or after the international filing date document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) document referring to an oral disclosure, use, exhibition or other means document published prior to the international filing date but later than the priority date claimed	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family
Date of the actual completion of the international search 15 October, 2001 (15.10.01)		Date of mailing of the international search report 23 October, 2001 (23.10.01)
Name and mailing address of the ISA/ Japanese Patent Office		Authorized officer
Facsimile No.		Telephone No.

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP01/08138

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 36
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Concerning the compounds obtained by the screening method as set forth in claim 36, it is completely unknown what particular compounds are involved in the scope thereof and what are not. Thus, the above claim is described in an extremely unclear manner. Such being the case, no meaningful opinion can be made on the novelty, inventive step and industrial applicability of the invention as set forth in the above claim.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.